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Genetic structure and antimicrobial resistance of foodborne *Escherichia coli* in Australia

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DECLARATION

I declare that the work contained in this thesis titled “Genetic structure and antibiotic resistance of foodborne *Escherichia coli* in Australia” is the outcome of my original research. To the best of my knowledge, this thesis contains results that are solely from this study except where due references have been provided. None of the work has been submitted for obtaining a degree to any other University.

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July, 2017

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"I can do all thing through Christ who strengthens me".

ABSTRACT

Poultry meat is thought to harbour the most "human-like" *E. coli* strains, capable of causing both intestinal and extra-intestinal infections in humans. Furthermore, poultry meat is also known to harbour multi-drug resistant *E. coli* strains. In this study, extensive sampling of retail poultry meat products sold in major supermarkets and independent butchers in Canberra was undertaken. Phenotyping and genotyping of the *E. coli* isolated from the meat samples was then performed to better understand the evolution and diversity of these strains and their significance to human health. Using a whole genome sequence based approach, the strains were then compared to clinical and commensal human *E. coli* isolates to determine if there is transmission between the two hosts.

A highly diverse population of *E. coli* in poultry meat was identified in this study. Although the exact contamination route is hard to determine, our findings suggest that poultry meat is frequently contaminated, not only with *E. coli* isolates from poultry birds but also significantly from other sources, possibly during processing chains and transport. In addition, the genetic structure comparison of human and poultry meat *E. coli* strains indicate that there is cross-transmission between the two hosts, but at low levels. Therefore, poultry meat serves as a low risk transmission route for zoonotic *E. coli* strains that can cause extra-intestinal infections. Our findings also suggest that the risk of exposure to intestinal or diarrheal pathogenic *E. coli* strains through poultry meat consumption and handling is even lower than exposure to extra-intestinal pathogenic *E. coli* (ExPEC) strains.

Furthermore, poultry meat in Canberra region does not serve as a highly significant carrier of antimicrobial resistant *E. coli* unlike several other countries. Resistance was commonly observed to antibiotic classes approved for use in poultry industries (like ampicillin and tetracycline), but was low for critically important antibiotics to humans which are banned in the industry (like fluoroquinolone and 3rd generation cephalosporins). In addition, no carbapenem and colistin resistance was observed, which has recently been identified in poultry meat products in many countries including China, United Kingdom, Denmark. Poultry meat in Canberra is therefore more frequently contaminated with *E. coli* strains from the birds rather than from other contamination sources.

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General Introduction

1.1 *Escherichia coli*

Escherichia coli (E. coli) is a rod-shaped, facultative anaerobic, non-sporing, Gram-negative bacterium of the family Enterobacteriaceae, of the phylum Gammaproteobacteria. The organism is part of the normal gut flora of warm-blooded organisms including mammals and some birds, and is commonly found in the lower intestine, i.e. caecum and colon (Kaper et al., 2004). Populations of *E. coli* are also found in water, soil, sediments, and food which form their secondary habitats (Leimbach et al., 2013). It is estimated that humans ingest approximately 10,000 cells of *E. coli* per gram of food we eat (Hartl & Dykhuizen, 1984), and faecal-oral transmission is the major route of entry for pathogenic strains of *E. coli* in humans (Madigan and Martinko, 2006, Tauxe, 1997). *E. coli* is highly heterogeneous and can range from commensal strains that are part of the normal gut flora, to highly virulent strains that are known to cause both intestinal and extra-intestinal infections (Levine, 1987, Kaper et al., 2004, Johnson and Russo, 2002). Intestinal pathogenic *E. coli* (IPEC) commonly referred as diarrheagenic *E. coli* are known to cause infections ranging from travellers' diarrhea to severe life-threatening infections like haemolytic uremic syndrome (HUS) (Levine, 1987, Nataro and Kaper, 1998).

Extra-intestinal pathogenic *E. coli* (ExPEC) strains can successfully colonize the gastrointestinal (GI) tract of healthy human hosts where they are part of the normal gut flora. When these *E. coli* strains leave their natural habitat (GI tract) and enter other organs or sites they are often responsible for causing infections like urinary tract infection (UTI), and more severe infections of the bloodstream, also known as septicemia or bacteremia, neonatal meningitis, pneumonia (especially in immunocompromised patients), sepsis and many other infections (Johnson and Russo, 2002, Smith et al., 2007). In fact, *E. coli* is the leading cause of UTI, known to cause more than 90% of UTIs worldwide (Foxman, 2010, Kucheria et al., 2005). In addition, an ExPEC subset, called avian pathogenic *E. coli* (APEC) are known to cause systemic infections like colibacillosis, a major problem of the poultry industry (Dho-Moulin and Fairbrother, 1999). APEC often closely resemble certain human ExPEC variants (Maluta et al., 2014),

which brought about the question do human ExPEC strains have food-animal reservoirs or even origin (Manges, 2016, Johnson et al., 2009).

1.2 Genetic diversity and evolution of *E. coli*

E. coli is a heterogeneous bacterium with a highly plastic genome. The core genome of a bacterial species is defined as the genes that are present in 'all' strains of a species. Genes of the core genome include the housekeeping genes that aid in processes like replication, transcription, and translation, thus make up the basic metabolic functions of bacterial species. The balance a bacterium's genome consists of those genes not present in every member of the species, and this is known as the variable genome (Touchon et al., 2009). Genes representing the variable genome are frequently associated with mobile genetic elements like plasmids, phages, and genomic islands, and often help bacterial isolates adapt to specific environmental conditions (Medini et al., 2005). The pan genome of a species consists of the core genome and all other genes found in the species. A typical *E. coli* genome, on an average, is around 5 MB in size and made up of about 4700 genes, of which about 2000 are common to all strains (the core genome), the balance of the genes (the variable genome) are drawn from a pool of genes numbering over 15,000 (Touchon et al., 2009). Although the typical *E. coli* genome is about 5 MB in size, genome size can vary by more than a 1 MB between strains and this is further evidence of the highly plastic nature of the *E. coli* genome.

Although mutation is the ultimate source of all genetic variation, the variation observed in a species represents the net effects of mutation, homologous recombination, together with horizontal gene transfer (HGT) (Reid et al., 2000, LeClerc et al., 1996). It has been estimated that a base in an *E. coli* genome is 100x more likely to be changed due to recombination than due to mutation (Touchon et al., 2009). However Touchon et al (2009) also demonstrate that this level of recombination is not sufficient to obscure the underlying phylogenetic relationships among strains.

1.3 Ecological structure of *E. coli*

The gastrointestinal tract or lower intestine of warm-blooded organisms is considered to be the primary habitat of *E. coli* (Savageau, 1983, Gordon, 2001). The organism can also be isolated from environmental samples like soil, sediment, and water, which are considered to be the secondary habitats, most often as a result of faecal contamination (Savageau, 1983, Gordon, 2001). Additionally, *E. coli* can be commonly isolated from retail food products including raw or cooked meats like chicken, turkey, beef, pork, and even from ready-to-eat foods like salads and vegetable dips (Johnson et al., 2005a, 2009). It is estimated that as much as half of the *E. coli* population spends their life cycle in secondary habitats rather than the habitat considered as 'primary' (Savageau, 1983). The presence and distribution of *E. coli* vary within and among hosts, population and region, and several factors including the host's diet, body size, gut morphology and climate serve as important predictors for the presence of *E. coli* (Gordon and Cowling, 2003, Escobar-Páramo et al., 2006, Blyton et al., 2014).

Resident strains of *E. coli* are defined as the 'persistent' strains which are capable of colonizing their respective hosts more effectively than other lineages of the same organism. Phylogroup B2 strains are thought to be more host-adapted than isolates of the other phylogroups and more likely to be resident (Nowrouzian et al., 2005, 2006). This ability may be due to the presence of certain virulence factors (VFs) like adhesins that aid in attachment (*papG*, *iha*), toxins (*hlyA, astA*), invasins (*ibeA, traT*) and siderophores or iron acquisition systems (*iroN, iutA*), which are often over-represented in lineages of this phylogroup (Nowrouzian et al., 2005, 2006, Gordon et al., 2005, Johnson et al., 2008). Also, these lineages are known to have tolerance to environmental stress conditions like pH and temperature. In addition, phylogroup B2 strains when present in a host have been shown to predominate over other phylogroups with limited co-occurrence (Adiba et al., 2010, Smati et al., 2013).

On the other hand, the transient strains of *E. coli*, those observed in a host once or on just a few occasions, often belong to phylogroups A and B1 (Nowrouzian et al., 2006). The strains from these phylogroups are generally seen to harbour fewer of the extra-

intestinal VFs that are common in phylogroups B2 and D (Johnson et al., 2001). Although, some commensal lineages like A-ST10, B1-ST155 strains have also been linked to carrying IPEC or ExPEC-related VFs (this study, Manges and Johnson, 2015, Skurnik et al., 2016), suggesting that some commensal groups may be as persistent in the host environment. The turnover of *E. coli* population in its primary human host environment was estimated to be two to four weeks for transient strains while resident strains turnover was over months or even years (Caugant et al., 1981).

1.4 Classification of *E. coli*

1.4.1 *Phylogenetic groups (Phylogroups)*

E. coli strains can be classified into eight phylogenetic groups (phylogroups), consisting of four major groups A, B1, B2, and D and four minor groups, namely C, E, F, and clade I (Clermont et al., 2013). Phylogroups B2 and D are considered to be the ancestral groups, and strains of these groups have a narrow host-spectrum, are highly host-adapted with larger genomes compared to strains of the other phylogroups (Johnson et al., 2001, Manges et al., 2001, Gordon and Cowling, 2003, Nowrouzian et al., 2005). Of note, a number of studies on commensal populations of *E. coli* have noted the high incidence of phylogroup B2 and D strains, especially in humans residing in temperate regions like France (Massot et al., 2016, Escobar-Páramo et al., 2004a) and Australia (Blyton et al., 2014, Gordon et al., 2015). On the other hand, phylogroups A and B1 are considered to be sister groups, which are made up of opportunistic commensals having broad host-spectrum (Duriez et al., 2001, Gordon and Cowling, 2003). Phylogroup A strains were often found to be over-represented in human populations residing in tropical regions (Escobar-Páramo et al., 2004a) and also in birds (Blyton et al., 2015), including poultry (Bonnet et al., 2009, Blyton et al., 2015, Wang et al., 2016, Obeng et al., 2012). Meanwhile, phylogroup B1 strains were often predominant in environmental samples including water (Power et al., 2005). Diarrheal *E. coli* strains are more common among phylogroups A and B1, although intestinal pathogens may also arise from phylogroups B2 and D (Okeke et al., 2010). Conversely, most *E. coli* isolated from extra-intestinal sites are members of phylogroups B2 or D, although strains belonging to phylogroups A and

B1 may also be extra-intestinal pathogens (Johnson et al., 2005a, Manges and Johnson, 2012).

The minor phylogroups of *E. coli* (C, E, F, and clade I) are not as well-characterized as the major phylogroups. Most studies still classify these groups as either A (for C groups) or D (for phylogroup F isolates) because the original Clermont phylogrouping method did not include these groups as part of the classification (Clermont et al, 2000). Phylogroup E and clade I overlap in their Clermont profiles when isolates are positive for *arpA*, *chuA*, *yjaA*. Consequently, until recently the only way of identifying strains belonging to the minor phylogroups was through multi-locus sequence analysis. The widely studied intestinal pathogen O157:H7 belongs to phylogroup E, and clusters closely with phylogroup B1 (Gordon, 2013). Phylogroup E strains were more common in animal faeces than human faeces, and especially more prevalent in the normal intestinal flora of healthy cattle (Clermont et al., 2011, Ferens and Hovde, 2011). Phylogroup F is related to phylogroups B2 and D, while phylogroup C strains are closely related to phylogroup B1 (Gordon, 2013). Certain lineages of phylogroup F (e.g., ST354, ST117) are also often associated with ExPEC and APEC-related infections in both humans and animals (Maluta et al., 2014), especially in companion animals (Guo et al., 2015) and poultry (Dissanayake et al., 2013, Mora et al., 2012). Phylogroup C strains are rare, but have been implicated as extra-intestinal pathogens (Maluta et al., 2014). Strains belonging to cryptic clade I (Walk et al., 2009) appear to be closely related to classical *E. coli* and appear to exchange genetic material more frequently with *E. coli* than with members of the other cryptic clades (Luo et al., 2011). The significance of clade I strains as pathogens is poorly understood.

Members of the 'genus' *Shigella*, represent at least four independently evolved lineages of *E. coli*. The 'subspecies', *S. flexneri*, *S. boydii* and *S. sonnei* are most closely related to phylogroups A and B1, while *S. dysenteriae* is most closely related to phylogroup D (Pupo et al., 2000). Only *Shigella* and EIEC, are considered obligate pathogens of humans while other groups are all considered facultative or opportunistic pathogens (Touchon et al., 2009).

1.4.2 *Serotypes or serogroups*

Since the early 1940's, the traditional typing method of *E. coli* was through serotyping or serogroup, first introduced by Kauffman (1943, 1947), and later revised by Ørskov and colleagues (1977). Serotyping was achieved by using antibodies against surface antigens, namely O-antigens targeting outer membrane somatic/ lipopolysaccharides (LPS) located in the chromosome, and H-antigens targeting flagellar surfaces (Fratamico et al., 2016). At present 186 O and 53 H antigens are recognised. A common example of an *E. coli* serotype is the widely studied pathogen O157:H7. The K-antigen typing that uses antisera which targets the capsule surface antigen of *E. coli*, though less popular, was also introduced and used since the late 1970s (Ørskov et al., 1977). When isolates were only O-antigen typeable, they were indicated as serogroups, common examples being the O1, O2, and O18 serogroups which are widely represented in ExPEC and APEC isolates (Moulin-Schouleur et al., 2007).

Often, many non-clinical *E. coli* isolates are O non-typeable, while most can be H typed unless they are non-motile strains lacking flagella (Feng et al., 1996). Recently with the advent of next generation sequencing, molecular or in silico serotyping is currently becoming more popular, as it has higher discriminatory and identification abilities (Joensen et al., 2015, Fratamico et al., 2016). Also, in silico serotyping using whole genome sequences are less prone to technical errors resulting from the cross-reactivity of antisera (Fratamico et al., 2016), and can be used for performing other screening like VFs (toxins) determination (Cheng et al., 2016). O-typing generally targets Wzy-dependent pathway which carries *wzy* (O-antigen polymerase) and *wzx* (O-antigen flippase) genes, and less commonly ATP-binding cassette (ABC) transporter-dependent pathway carrying *wzm* (O-antigen ABC transporter permease) and *wzt* (ABC transporter ATP-binding) genes (Greenfield and Whitfield, 2012). Meanwhile, H-typing targets the *fliC* gene which is encoded by the flagellar filament structural protein called FliC, and additionally *flnA, flkA, flmA* genes for 9 H-types of non-*fliC* flagellin genes (Joensen et al., 2015, Wang et al., 2003). Although, serotyping/ serogrouping of *E. coli* does not provide specific phenotypic traits as they can be highly variable, with a particular lineage (say, ST117) having more than 5 or more serotypes.

1.4.3 *Multi-locus Sequence Typing (MLST)*

Multi-locus sequence typing or MLST is a widely used typing technique for *E. coli* and other species. The method is based on sequencing 400-500 bp fragments of 6 – 8 housekeeping genes (core genome genes) (Clermont et al., 2015). Every unique combination of alleles is designated as a sequence type (ST). Until the advent of whole genome sequencing MLST was considered to be the best typing method for *E. coli*, as it provides reliable phylogeny with high discriminatory power (Sahl et al., 2012, Tartof et al., 2005).

Three MLST schemes are available, namely the Achtman scheme (Wirth et al., 2006) hosted by Warwick Medical School (Coventry, UK), Institut Pasteur scheme (Jaureguy et al., 2008) hosted at Institut Pasteur (Paris, France), and Whittam scheme (Reid et al., 2000) hosted by Michigan State University (East Lansing, USA). Each of the schemes are based on different housekeeping gene combinations, although the *icd* gene is common to all schemes (Clermont et al., 2015).

In this project, isolates were assigned to an ST using the Achtman and Institut Pasteur schemes, and referred to by their Achtman scheme ST. In addition, strains with STs that differed by a single MLST locus (of any housekeeping gene) were defined as members of same clonal complex (CC) (Coque et al., 2008, Blanco et al., 2011).

1.4.4 *Other Molecular characterization techniques of E. coli*

Repetitive Element Palindromic (REP)-typing

REP-typing is a PCR-based DNA fingerprinting technique used to determine strain richness in a sample of isolates and is also used to determine strain relatedness (Olive and Bean, 1999). ERIC (Enterobacterial Repetitive Intergenic Consensus) (Versalovic et al., 1991) and $(CGG)_4$ (Adamus-Bialek et al., 2009) are two commonly used REP typing primers. The discriminatory power of these two primers often differs, depending on the nature of the sample, although, CGG-based PCR is often considered to have higher

differentiating ability than ERIC-PCR (Adamus-Bialek et al., 2009). Consequently, both primer sets are often used together to differentiate strains (Blyton et al., 2013).

Pulsed-Field Gel Electrophoresis (PFGE)

PFGE is another DNA fingerprinting technique that was first introduced by Schwartz and Cantor in 1984, and it is highly discriminatory. It is typically used for comparing bacterial isolates in epidemiological studies in order to identify and tracking outbreak. PFGE is gradually losing popularity as it is a time-consuming and expensive procedure (Malathum et al., 1998). As for REP-typing techniques, it is not specific for *E. coli* and can be used for typing other bacterial species.

Randomly Amplified Polymorphic DNA (RAPD)

RAPD is a PCR-based DNA amplification technique also used for strain characterization (Williams et al., 1990). It amplifies random segments of genomic DNA and typically produces smaller DNA fragments than REP-PCR typing. The RAPD technique is less time-consuming and relatively inexpensive compared to other typing methods but does not have as high discriminatory power (Sabat et al., 2013).

1.4.5 *Whole genome sequencing*

Whole genome sequencing (WGS) is increasingly popular as prices have decreased and library preparation methods have been simplified. WGS is now routinely used as a typing technique in a number of laboratories worldwide. It has proven to be vastly useful especially in the cases of epidemiological studies, where strains are required to be traced back to their origin to prevent further outbreaks and spread. The platform was aptly referred to as the ideal “gold standard” in phylogenetic studies (Leimbach et al., 2013) in its ability to provide a common platform for researchers all over the world. It also gives the option of easy access to genome data for countries with limited facilities and funds. The use of WGS as a routine screening procedure of microorganisms and eliminating the many other molecular typing techniques may not currently be

achievable to many research groups, but is definitely worth considering as a future approach (Dale and Woodford, 2015). Not only does it help in phylogenetic classification, it enables *in silico* characterization, such as, multi-locus sequence typing (MLST), serotyping, plasmid replicon typing, VF content, resistance gene determinants, and also pan- and core-genome contents can also be inferred (Clermont et al., 2015, Vangchhia et al., 2016). These methods provide a high throughput microbial epidemiology study platform that can in turn help in improving diagnosis, surveillance studies, tracing pathogens, and in studying antimicrobial resistance (AMR) dissemination (Singer, 2015, Wyrsch et al., 2016).

A number of simplified Bioinformatics tools like Mauve (Darling et al., 2010), CLC Genomics Workbench (CLC Bio Aarhus, Denmark), Harvest (Treangen et al., 2014) are available. Moreover, web-based applications like BLASTN suite (https://blast.ncbi.nlm.nih.gov) hosted by NCBI (National Center for Biotechnology Information), CGE (http://www.genomicepidemiology.org/) (Larsen et al., 2012), PATRIC (https://www.patricbrc.org/) (Wattam et al., 2014), SEED (http://pubseed.theseed.org/) (Overbeek et al., 2014), CARD (https://card.mcmaster.ca/) (McArthur et al., 2013), PHAST (http://phast.wishartlab.com) (Zhou et al., 2011), are also easily available and accessible, even with limited bioinformatics skills.

1.5 Poultry farming: From farm to consumption

1.5.1 *The poultry industry*

Chicken meat is now the most consumed meat in Australia, with an average annual consumption of 46.2 kilograms per person, and consumption is continuing to increase (ABARES, 2016). The popularity of the meat is largely due to its affordability compared to other meats like beef and pork, and partly because of its nutritional value, as it serves as a good source of low-fat meat protein (Charlton et al., 2008). In Australia, all fresh chicken meat is from locally reared and grown poultry with very limited imports of processed chicken meat products (Australian Chicken Meat Federation ACMF,

http://www.chicken.org.au). It is estimated that approximately 600 million meat chickens are slaughtered annually in Australia, producing about 1 million tonnes of meat (ABS, 2016). The chicken meat industry is highly vertically integrated, and individual companies own almost all aspects of production - breeding farms, multiplication farms, hatcheries, feed mills, some broiler growing farms, and processing plants. Australia's meat chicken is largely supplied by two privately owned companies, Baiada and Inghams Enterprises, which supply more than 70% of Australia's broiler chickens. The remainder of the meat market is supplied by another six medium-sized, privately owned companies, with each supplying approximately between 3% and 9% of the national market (ACMF).

Rearing broiler chickens, from day old chicks to the day of processing, is generally contracted out by processing companies to contract growers. About 800 growers produce about 80% of Australia's meat chickens under these contracts, with the balance produced on farms owned by the major producers. In Australia, conventionally reared meat chickens are reared in sheds (commonly 150 metres long and 15 metres wide) usually with temperature and ventilation control systems and are free to move around. Free range meat chickens have access to outdoor range and indoor shelter. Chickens are then reared for approximately 35-55 days for conventional and free range meat chickens, and 65-80 days for organic meat chicken. Conventionally reared poultry accounts for about 90% of the birds reared for production, while free range meat chickens make up most of the balance of production with organic chicken representing less than 1% of total production (ACMF website).

The processing of poultry involves two main stages, each with a number of steps, some of which can be quite vigorous and complex. The complex processing steps explains why bacterial contamination is often higher in poultry meat products as compared to other meats like pork and beef (Vincent et al., 2010, Johnson et al., 2003a, 2005a).

After slaughter, the feathers are removed and the bird is eviscerated, then the carcasses are left whole or cut into pieces. The chilling cum washing step involves dipping the meat

products in a mixture of water and ice to chill them below 5^{o}C, which is usually sanitized with chlorine at levels of 3-5 ppm to prevent and contain microbial contamination. This step is carried out for all types of chicken meat unless marked as 'chemical free' in Australia (ACMF, 2011). After these steps, the raw meat is then chilled or frozen, packaged for distribution and then transported to supermarkets, butchers, restaurants, food services, pet food manufacturers, or sent for further processing to the second stage. The second stage is optional, where the meat undergoes further processing by getting coated, crumbed, completely or partially cooked, or addition of other dressings. Only about 31% of meats undergo second stage processing while approximately 69% directly gets distributed after primary processing and chilling.

1.5.2 *Poultry meat and E. coli*

One of the most significant findings of microbiological examination of food worldwide is the high frequency of bacterial contamination of many food products, including poultry meat (Johnson et. al., 2005a, Obeng et al., 2012, Manges et al., 2012). The bacterial contamination can range from food poisoning-inducing bacteria like *Salmonella* and *Campylobacter* species, to more clinically common bacteria like *E. coli*. Undercooked meat, especially poultry meat, is a widely emphasized food safety concern with the meat undeniably being non-sterile (Yang et al., 1998). Though the expected reaction would be hygiene and care during handling and cooking, studies conducted on food-handling behaviours have indicated that some populations do not understand the seriousness of unhygienic practices (Yang et al., 1998, Jones and Schaffner, 2005). Moreover, highlighting on ideal handling practices for poultry processing plants' workers and butchers is uncommon, considering the fact that handlers and workers could potentially cross-contaminate the meat or vice-versa (Jones and Schaffner, 2005). The chance of opportunistic organisms like poultry-associated *E. coli* strains gaining entry to humans from poultry meat, or human-associated *E. coli* strains contaminating poultry meat is quite high.

Worldwide, *E. coli* is known to cause more infections than *Salmonella* and *Campylobacter* combined (WHO, 2012). However, it is also important to understand that

not all infections that arise from consumption of contaminated food directly lead to food poisoning (Jones and Schaffner, 2005). The association of poultry meat with ExPEC is not as straightforward as assessing meat with diarrheal pathogens that may cause food poisoning. Unlike IPEC strains which induce diarrheal symptoms that usually arise rapidly, other strains of *E. coli* do not induce intestinal complications even after consuming/handling contaminated food. Although they may subsequently cause extra-intestinal infection, it is much more difficult to trace the source of such strains (Kaper et al., 2004).

In humans, mostly because of the food we consume, it is estimated that there is a great turnover of *E. coli* strains daily in the GI tract (Collignon, 2009). This suggests that the possibility of ingesting *E. coli* strains from food source like undercooked chicken meat is rather high, and that these strains may harbour the genes responsible for virulence and/ or resistance. These strains in poultry meat may not only be capable of causing diarrhea or serve as a zoonotic reservoir of food, but also serve as a potential reservoir and transmitter of antimicrobial resistance (Manges, 2016). Although this hypothesis is plausible, it is challenging to determine the exact transmission route, especially for ExPEC and AMR strains. Studies conducted by Johnson et al. (2005a, 2005b) indicated that retail poultry meat was found to be more similar to human UTI causing isolates than other meats like pork, beef, and other foods, and also exhibited higher levels of antimicrobial resistance and virulence mechanisms. As a result, it is indicated that a food reservoir especially in the form of poultry meat, is almost certainly present for ExPEC and AMR (Manges, 2016, Johnson et al., 2005a, 2005b, 2003).

1.5.3 *Antimicrobial use in poultry industry, Australia*

Australia is distinctive with regards to the judicious use of antimicrobials in food-producing animals, including poultry. The highly controversial use of antimicrobials as growth promoters, especially in broilers destined for human consumption, has been banned for over thirty years, unlike countries like China, where such a ban has still not been successfully implemented (Collignon, 2015). Also, the use of important antimicrobial classes, like fluoroquinolone, in agriculture has never been approved in

Australia (Cheng et al., 2012). Antimicrobials are used solely in the poultry industries either for prophylactic purposes i.e. for the prevention of outbreaks of diseases, or for therapeutic purposes to cure sick birds.

The antimicrobial classes approved for use in Australian poultry farms are penicillins (amoxicillin, ampicillin), tetracyclines (chlortetracycline, oxytetracycline, tetracycline), aminoglycosides (neomycin, framycetin, spectinomycin, apramycin), sulfonamides (sulfadoxine, sulfamethazine, sulfaquinoxaline), quinoxalines (olaquindox), nitroimidazoles (dimetridazole, ronidazole), macrolides (erythromycin, kitasamycin, tylosin, oleandomycin), lincosamides (lincomycin, spectinomycin), streptogramins (virginiamycin), flavomycin (bambermycin), polyethers (lasalocid, maduramycin, monensin, narasin, salinomycin, semduramycin) and polypeptides (bacitracin, gramicidin). Also, sulfonamide/ trimethoprim combinations are used for broad-spectrum infections as primary agents in oral and injectable forms. The use of macrolides (tylosin, oleandomycin, and kitasamycin) and streptogramin (virginiamycin) has been under review since 2001 by the APVMA (Australian Pesticides and Veterinary Medicines for Agricultural and Veterinary Chemicals), as per recommendations by the Joint Expert Technical Advisory Committee on Antibiotic Resistance (JETACAR) report in 1999 (http://www.health.gov.au/, accessed on 6th October, 2016). Avoparcin, belonging to glycopeptide class of antibiotic was banned for use in livestock feed including poultry since 2006, as it has a similar chemical structure with vancomycin, a first-line treatment used against methicillin-resistant *Staphylococcus aureus* (MRSA) infections in humans. It was initially used widely in Australia and the European Union as a prophylactic for preventing necrotic enteritis in poultry, but raised concern as widespread use could potentially lead to increasing prevalence of vancomycin-resistant bacteria especially after the rapid rise of vancomycin-resistant enterococci (VRE).

It can be noted that in the poultry industry, the use of broad-spectrum antibiotic classes or antibiotics used for treating human infections caused by Gram-negative bacteria like *E. coli*, are not approved (http://www.chicken.org.au/files/ACMF_Antibiotics_Policy.pdf, accessed on 29th

September, 2016). The standout exception would be the use of sulfonamide/ trimethoprim combination drug, which is used in the form of sulfamethoxazole/ trimethoprim combination in humans for treating common infections like urinary tract infection (UTI). This cannot be ruled out as a concern because the rise of resistance to both sulfonamides and trimethoprim is evident, as also reflected in this study with 23.7% (sulfonamide) and 23% (trimethoprim) of isolates exhibiting resistance. In addition, the fact that the resistance gene determinants of these antibiotic classes are located in mobile genetic elements (plasmids) heightens the concern, as the genes can be readily transferred from one strain to another, or even from one species to another thus transmissible to humans.

1.5.4 *Antimicrobial resistance*

A major worldwide threat is the rise in antimicrobial resistance, where resistance is now developing faster than the development of new antimicrobials (Sanders, 2001). The use of antimicrobials not only in humans but more extensively (approximately 70%) in food-producing animals is a real concern (WHO, 2012). Resistance now exists to virtually every antimicrobial class including last-resort antimicrobials belonging to classes like 3rd and 4th generation cephalosporins, fluoroquinolones, carbapenems, and the recently identified colistin resistance (Collignon, 2015, Doumith et al., 2016).

The main mechanisms by which a strain achieves resistance is by chromosomal mutation and by the acquisition of mobile genetic elements like plasmids and bacteriophages (Holmes et al., 2016). Harmless bacteria in the gut can also serve as a reservoir of antibiotic resistance by participating in HGT. The concern is elicited when resistance markers are carried in mobile genetic elements like plasmids, through which they can quite easily be carried from one strain to another or even from one species to another, through HGT. This does not rule out the significance of chromosomal-mediated resistance as *E. coli* is known to have high chromosomal plasticity, whereby the organism can adapt to different levels of selective pressures through mutation (Touchon et al., 2009, Skurnik et al., 2016). Also, vertical transfer is another significant source of resistance gene transfer, whereby whole bacterium carrying resistance genes gets

transferred (Ingram et al., 2013). Resistance markers in *E. coli* genome are often co-located on the same plasmid or integron gene cassettes thus giving rise to multi-drug resistance (Barton et al., 2003, Fortini et al., 2011, Szmolka and Nagy, 2013, Ingram et al., 2013). Also, a single mutation or resistance marker can confer resistance to two or more antimicrobial classes, as in the case of *aac(6')-Ib-cr*, which confers resistance to both aminoglycoside and fluoroquinolone classes (Ingram et al., 2013). This is termed as co-selection where the use of one antimicrobial can select for resistance to another antimicrobial class (Gyles, 2008, Ingram et al., 2013).

In September 2016, the United Nations (UN) General Assembly called a 'high-level meeting on antimicrobial resistance', which is only the fourth time in history when a UN General Assembly was called for public health related issue (WHO, 2016), with the previous three being for HIV, non-communicable diseases, and Ebola. Back in 2011, World Health Organization (WHO) had declared that antimicrobial resistance is a growing threat and serious global concern. A 'One Health' approach in the judicious use of antimicrobials in both humans and animals is needed for promoting best practices to avoid and delay the spread and emergence of antimicrobial resistance (WHO, 2016). It is estimated that by 2050, 10 million people will die from multi-drug resistant infections annually, if resistance keeps spreading and developing at the same rate as now and also if no new antibiotics are not developed.

In poultry farms, antimicrobials are not only used as therapeutic agents for treating sick birds but more often as prophylactics for preventing outbreaks of diseases. Often, an entire population of the poultry flock are exposed to these antimicrobials directly by introducing the agents to their drinking water or feeds, rather than only treating sick birds which then selects for antimicrobial resistant microorganisms like *E. coli* (Diarra et al., 2007, Barton and Wilkins, 2001). Even though this strategy is to avoid as much as possible the incidence of outbreaks which often wipe out entire flocks, it adversely serves as a breeding ground for resistant bacteria (van den Bogaard et al., 2001). Furthermore, the use of specific antimicrobial classes (like tetracycline) has directly been linked to the occurrence and selection of resistance to the same classes (Miranda et al.,

2008), particularly in poultry industries where antimicrobials are used so extensively (Aarestrup, 2015, van den Bogaard and Stobberingh, 2000). A resistant strain harboured by a bird can easily be transmitted from one bird to another, especially in farms where the birds are reared in close proximity. As indicated by Aidara-Kane et al., 2013, resistant bacteria replace the susceptible ones which then circulate to other hosts and are also capable of transferring resistance genes to pathogens. Use of antimicrobial agents for non-therapeutic purposes including use as prophylactics has also been raised as a concern, with unsuccessful proposals for a complete ban (Barton and Wilkins, 2001).

Although Australia has successfully banned the use of antimicrobials as growth promoters for more than decades now, they are still largely used as both prophylactics and therapeutics (Obeng et al., 2012). In countries like Greece and China, critically important antimicrobials to humans like fluoroquinolones are still controversially used widely not only as prophylactics but also as growth promoters (Gousia et al., 2011, Collignon, 2009, Price et al., 2007, Krishnasamy et al., 2015). Several studies indicated that APEC isolates were often multi-drug resistant (MDR), even to important antimicrobials like fluoroquinolones (Zhao et al., 2005) and extended spectrum beta-lactamases (ESBL) (Solà-Ginés et al., 2015), and were also described to closely resemble human ExPEC variants (Mellata, 2013). The fact that these strains not only serve as potential zoonotic organisms to humans but are also often coupled with MDR, is a great threat not only to the poultry industry but also to public health. Also, human deaths were associated with the excessive antimicrobial use in poultry in Netherlands, Europe (Collignon et al., 2013, Overdevest et al., 2011, de Kraker, 2011). These MDR patterns observed in other parts of the world is nevertheless relevant to Australia because these determinants may spread to human lineages through international travels and migration (Kennedy and Collignon, 2010). Therefore, both poultry and meat products serve as significant reservoirs of antimicrobial resistance.

Moreover, human and poultry meat *E. coli* isolates are often observed to harbour similar resistance gene determinants (Aliyu et al., 2016, Koga et al., 2015, Leverstein van-Hall et al., 2011). This further highlights the importance of conserving and sensible

antimicrobial usage, not just in humans but all the more in food-producing animals, as the potential resistant and often virulent strains end up colonizing humans anyway (Manges et al., 2007, Johnson et al., 2005a, b). The exact route of entry is often hard to determine, especially in the cases of poultry associated products, where they undergo many processing steps, packaging, and transportation before it finally reaches humans for consumption. Nevertheless, the importance of finding these potential zoonotic strains in both poultry meat products and humans cannot be underestimated, as either through consumption or handling, humans associate with the meat directly or indirectly, thus cross-contaminating. If anything, it should be considered as a challenge to trace back to the exact point source not just for epidemiological purpose but also for awareness, to better understand the importance of hygiene and know what the food we consume could potentially cause or is already causing.

1.6 Extra-intestinal pathogenic *E. coli* (ExPEC) and avian pathogenic *E. coli* (APEC)

Extra-intestinal pathogenic *E. coli* (ExPEC) can be broadly defined as the pathogenic strains isolated from infection sites in hosts, outside of the intestine or gastrointestinal (GI) tract (Russo and Johnson, 2000, Köhler and Dobrindt, 2011, Smith et al., 2007). Additionally, the ExPEC counterpart in poultry is referred to as avian pathogenic *E. coli* (APEC). The intestine of humans and birds can serve as a reservoir of ExPEC and APEC strains respectively, where they maybe asymptomatically present as part of the regular gut flora. These strains are often capable of causing infections once they leave their resident site, and are known for causing infections like UTI, neonatal meningitis, septicemia and even septic shock in humans (Smith et al., 2007), and systemic infections like colibacillosis in poultry (Dho-Moulin and Fairbrother, 1999).

APEC is considered to be the leading cause of poultry birds' mortality and infections, in the form of severe respiratory diseases like colibacillosis leading to aerosacculitis, often followed by systemic infections like septicemia, and also arthritis, cellulitis, osteomyelitis, and death (Dho-Moulin and Fairbrother, 1999, Rodriguez-Siek et al., 2005, Dziva and Stevens, 2008, Barbieri et al., 2013, Braga et al., 2016). Outbreaks

caused by APEC are a huge threat and problem for the poultry industry, and is estimated to cost significant economic losses to major poultry industries worldwide (Gyles, 2008, Barnes et al., 1997). To counter outbreaks caused by organisms like APEC from arising, the birds are often fed with antimicrobials as a prophylactic. In addition, a large number of antimicrobials are used for therapeutic purposes in cases where the prevention doesn't work and birds are already sick (Barton et al., 2003). The farm environment can thus serve as breeding ground/ pool for highly virulent and resistant strains of *E. coli* (Cortés et al., 2010). Furthermore, APEC has also been linked to human ExPEC, and often belong to the same clonal groups (like ST95, ST117) as those causing extra-intestinal infections in humans (Maluta et al., 2014, Mora et al., 2012, Moulin-Schouleur et al., 2007, Rodriguez-Siek et al., 2005). In other words, APEC is rightly classified as a prototype of ExPEC in birds (Bélanger et al., 2011) and as such, a widely focused study subject compared to ExPEC-associated infections in other livestock like cattle (Wu et al., 2012).

The ability of *E. coli* to cause extra-intestinal infections is often associated with its VFs content and is site specific (Johnson et al., 2001). The common VFs in ExPEC strains are adhesins and fimbriae (*afa/draB, papAH, papC, papG, fimA, fimH, focG, hra, iha, tsh, sfa/foc, upaG*), invasins (*ibeA*, *tia, kpsE*), aerobactins and iron-acquisition systems (*iutA, iucC, iroN, ireA, chuA, fyuA, sitA*), toxins and hemolysin (*vat*, *usp, hlyA*), host defence systems (*nleB*), serum resistance and survival or protectins (*neuC, ompT, traT, iss*), and other factors like autotransporters (*cah*, *pic, sat*) and bacteriocins (*cva, cia, cma, cba*) (Köhler and Dobrindt, 2011, Smith et al., 2007, Johnson et al., 2001). Combinations of these factors are collectively termed as ExPEC-related VFs (Mora et al., 2009, Johnson and Russo, 2005), although their presence does not always lead to infections nor indicate pathogenicity. These VFs are usually located on genomic islands termed as pathogenicity-associated islands (PAIs) (Hacker et al., 1997, Diard et al., 2010). Resident strains in the human gut, especially of phylogroup B2 are often known to harbour high VFs without causing any harm or infections, and thus classified as asymptomatic opportunistic strains (Gordon et al., 2005, Blyton et al., 2014). Commensal *E. coli* strains which are naturally present in the large intestine (commonly in the caecum and colon) belonging to the non-B2 phylogroup may be displaced by the more host-adapted

phylogroup strains (say, B2-ST131) (Diard et al., 2010). As a result, ExPEC is considered to be a consequence of commensalism, by which strains that were asymptomatic can cause infection once they leave their resident site (Diard et al., 2010). This thin line between commensalism and pathogenicity thus often makes it hard to distinguish between commensals and ExPEC strains (Diard et al., 2010, Köhler and Dobrindt, 2011).

A study by Johnson et al. (2003a) indicated that when strains harbour two or more of *sfa/focDE*, *afa/draBC*, *papA/C*, *kpsMT* II, and *iutA*, they are considered to be capable of causing ExPEC infections, and termed the strains harbouring these genes as having ExPEC trait/ status. So far, no single VF has been linked to infection, but rather the VFs act in combination (Dale and Woodford, 2015). Virulence is rightly considered to be a multifaceted process that results from a number of genetic combinations (Tourret et al., 2010). In this study, poultry meat harbouring two or more of these VFs were thus termed as having ExPEC status, although ExPEC status cannot be limited only to strains harbouring these factors (Johnson et al., 2003a) nor are such strains definitely pathogenic (Diard et al., 2010).

Even though a number of VFs associated with APEC are well-defined, it is hard to point to exact factors as responsible for causing APEC infection. VFs like *tsh, traT, cva*, *iss*, *iutA*, *irp2*, *hlyA/F* which mainly aid in adhesion and iron transport systems were often over-represented in APEC organisms recovered from infection sites (Mellata et al., 2003, Ewers et al., 2004, 2007, Johnson et al., 2008, Bonnet et al., 2009, Maluta et al., 2014, Mora et al., 2012, Solà-Ginés et al., 2015). A study by Johnson et al. (2008) indicated that APEC strains were more likely to harbour VFs like *iutA*, *hlyF*, *iss*, *iroN* and *ompT* than avian faecal *E. coli* (AFEC) (Johnson et al., 2008, Solà-Ginés et al., 2015). The genes were often present in the colicin V plasmid, hence presence of colicin V (*cva*, *cvi*) is often associated with the zoonotic ability of APEC isolates (Johnson et al., 2007). Although, the presence of these specific virulence genes in strains isolated from non-infection sites does not necessarily confirm the pathogenesis of strains, but rather points to the fact that opportunistic lineages harbouring VFs often associated with ExPEC and APEC infections are present.

Majority of ExPEC isolates in humans are predominantly linked to phylogroups B2, D and F (Manges et al., 2001, Zhang et al., 2002, Johnson et al., 2003b, Kanamaru et al., 2006) and less so to phylogroups B1, A, C and E (Maynard et al., 2004, Manges and Johnson, 2015). On the other hand, the lineages covered by the term APEC can be very broad ranging from the 'pathogenic' group B2 and D's, to the 'commensal' sister groups B1 and A (Wu et al., 2012, Mellata, 2013).

1.7 Poultry meat as reservoir of ExPEC and APEC

The meat we consume is sometimes contaminated with *E. coli* strains not only from the birds but also with human-associated lineages (like B2-ST131) (Manges, 2016). Quite a lot of studies have indicated that poultry, both birds and meat products, harbour strains similar to infection-inducing APEC strains (Johnson et al., 2008), but also human uropathogenic *E. coli* (UPEC) (Jakobsen et al., 2010, 2012, Vincent et al., 2010), neonatal meningitis *E. coli* (NMEC) (Ewers et al., 2007, Tivendale et al., 2010), sepsis-associated *E. coli* (SEPEC) (Mitchell et al., 2015), collectively called as ExPEC (Russo and Johnson, 2000). Though sick birds in poultry farms are technically not expected to make it to meat processing plants, evidence from different studies sometimes suggest otherwise (Johnson et al., 2009, Moulin-Schouleur et al., 2007). Although, these APEC-related isolates could have also come from the birds' intestine, where they were part of the normal flora, as occurs for ExPEC-related strains in human gut. The fact that these meat products meant for human consumption sometimes carry organisms with potential virulence and resistance may serve as a food safety issue. It is hard to trace back the exact source from which these potential APEC organisms arise, but most likely originate from the birds itself, though humans as a source of contamination can neither be ruled out (Aslam et al., 2014). Previous studies have indicated that avian faecal *E. coli* (AFEC) are often quite different from APEC, mostly in terms of their VF content (de Brito et al., 2003, Johnson et al., 2012, Solà-Ginés et al., 2015). Another potential source from which these organisms can enter is through feed (Hofacre et al., 2001, Sapkota et al., 2007), environmental sources like water fed to the birds and soil in farms (Blaak et al., 2015). The lineages widely isolated from human ExPEC and poultry APEC infections include clonal groups belonging to STs 131, 95, 69 and to a lesser extent, STs ST10 and ST117

(Manges, 2016, Day et al., 2016, Manges and Johnson, 2015). Most of these lineages, apart from harbouring high VFs were also commonly MDR (Day et al., 2016, Manges and Johnson, 2015).

Epidemiological studies conducted in specific human populations noted that highly similar strains were isolated from geographically matched human infection samples and poultry meat (Jakobsen et al., 2012, Manges et al., 2007, Johnson et al., 2005a, b). These studies used traditional molecular typing methods like pulsed-field gel electrophoresis (PFGE) (Jakobsen et al., 2012, Manges et al., 2007) and random amplified polymorphic DNA (RAPD) (Johnson et al., 2005a) to determine strain similarities. Evidence from these studies thus suggest that the food we consume contributes, to some level, in transferring ExPEC-related isolates by cross-contamination, and also that food-producing animals serve as ExPEC reservoir (Manges, 2016, Mitchell et al., 2015, Clermont et al., 2011, Leverstein van-Hall et al., 2011, Jakobsen et al., 2010, Ramchandani et al., 2005, Johnson et al., 2003a). A limitation of these studies is they have been based on similarities in their ST membership and or similarities in their VF and/ or resistance profiles (Singer, 2015). A WGS-based study by de Been et al. (2014) made observations quite different from the molecular-based epidemiological studies. Even though some strains isolated in poultry meat were of the same ST as the human ExPEC strains (like STs 95, 131, 69), shared serotypes, harboured similar VFs, and sometimes resistance genes as well, most often they were not identical and clustered separately from human isolates. An extensive review on foodborne ExPEC by Singer (2015) rightly suggested that studies with better experimental design and preferably WGS approach is required for transmission route, source tracing and comparative studies.

1.8 Intestinal pathogenic *E. coli* (IPEC) and poultry meat

Intestinal pathogenic *E. coli* (IPEC) are responsible for causing mild diarrheal infections to severe infections like haemolytic uremic syndrome (HUS) in humans and livestock. IPEC variants include enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E.*

coli (EAEC), diffusely-adherent *E. coli* (DAEC), and adherent invasive *E. coli* (AIEC) (Kaper et al., 2004). Shiga-toxin *E. coli* (STEC) or verotoxigenic *E. coli* (VTEC) is another type of IPEC which is a sub-type of ETEC/ EHEC, differentiated by the presence of toxin *stx*$_1$ and *stx*$_2$ (Kaper et al., 2004, Paton and Paton, 1998, Kramarenko et al., 2016). Some IPEC groups like EHEC (E.g. O157:H7) are additionally known to have very low infectious dose (100 to 200 cells) and considered obligate pathogens, while other sub-types like ETEC and EPEC have a comparatively high infectious dose (10^8 to 10^{10} cells) (Nataro and Kaper, 1998). IPEC strains are capable of successfully colonizing the mucosal surface of GI regardless of their competition with resident flora strains (Nataro and Kaper, 1998). Diarrheal pathogens are still responsible for causing high rates of morbidity and mortality worldwide, especially in developing countries with poor hygiene and limited clean basic necessities like water and food (Croxen et al., 2013, Gomes et al., 2016, Manning et al., 2008).

Intestinal pathogenic *E. coli* (IPEC) are often associated with VFs distinctly different from those of ExPEC strains, though exceptions do exist (Reid et al., 2000, Escobar-Páramo et al., 2004b). The common examples are genes like *eaeA* (intimin), and *stx*$_{1\text{-}2}$ (Shiga toxin) which are largely only over-represented in IPEC groups like EPEC, ETEC, EHEC, and STEC strains that cause intestinal infections (Paton and Paton, 1998, Kaper et al., 2004). Although, the same does not apply for all VFs, like iron transport genes (E.g. *chuA*, *iucC*). Certain STs also have a link in determining whether strains are of IPEC (like ST11 for O157:H7) or ExPEC (like ST131) lineages to an extent, although some STs like ST10 have been associated with both intestinal and extra-intestinal infections (Olesen et al., 2012, Gomes et al., 2016). There are some studies which have also indicated that certain ExPEC-related and ExPEC infection inducing strains like UPEC and APEC strains also carry VFs responsible for causing intestinal infections like diarrhea (Stacy et al., 2014, Markland et al., 2015, Abe et al., 2008). Additionally, characterized IPEC sub-groups like EAEC have also been linked to cause UTI and bacteremia (Olesen et al., 2012, Herzog et al., 2014).

The consumption of undercooked meat is a significant and serious cause of food poisoning (Doyle, 1991). The presence of highly opportunistic IPEC strains, including obligate pathogens like O157:H7, in food meant for human consumption, is a significant health concern and therefore is studied widely. Although the association of IPEC with poultry meat is not as common as ExPEC's association with poultry meat, it is not non-existent as indicated in a number of studies worldwide (Chinen et al., 2009, CDC Report, 2010, Lefebvre et al., 2008, Alonso et al., 2012, Comery et al., 2013, Cabal et al., 2013, Ahmed and Shimamoto, 2014, Hoang Minh et al., 2015). Strains harbouring EPEC-related VFs like *eaeA* (Oh et al., 2012, Alonso et al., 2012), and STEC/ EHEC genes like stx_1, stx_2 were detected (Hoang Minh et al., 2015, Kagambèga et al., 2012). Also, O157:H7 strains were isolated in both birds and carcasses meant for meat (Lefebvre et al., 2008, Chinen et al., 2009, Cabal et al., 2013). Fertilizers and manures obtained from poultry farms containing poultry litter has also been associated to harbour potential diarrheagenic *E. coli* strains (Puño-Sarmiento et al., 2014). The lower prevalence of foodborne outbreaks linked to IPEC contaminated chicken could be due to the absence of such groups in the intestinal flora of healthy poultry (Ferens and Hovde, 2011).

1.9a Research Aims

While there have been several studies focusing on *E. coli* diversity in poultry meat, so far to the best of our knowledge there are no comprehensive studies that have been carried out in Canberra region. Although some studies have compared conventional to organic to free range or compared meat types, few have taken a very stratified sampling approach. The aim is to carry out an extensive study comparing *E. coli* isolated from retail meats of different types (whole meat, mincemeat, thigh fillet, breast fillet, and wings), sampled during summer, autumn and winter seasons. In addition, breast fillet produced from poultry reared under different husbandries, namely conventional, free range, and organic will also be studied and compared. The meat samples will be collected from the major supermarkets and independent butchers located in three of the main town centres (Belconnen, Gungahlin and Tuggeranong) in Canberra, Australian Capital Territory, Australia. Phenotypic and genotypic characterization will be carried out on all the *E. coli* isolated from all meat samples.

All unique strains will then be characterized for their antimicrobial susceptibility profile to determine their resistance traits. Additionally, whole genome sequencing (WGS) will be performed for a subset of strains representing each phylogroup. WGS-based approach will be used for studying the genetic structure and diversity of *E. coli* in poultry meat. For this study, WGS data will then be used for in silico characterization including multi-locus sequence typing (MLST), serotyping, and for determining virulence factors (VF) content, chromosomal and plasmid-mediated antimicrobial resistance gene carriage.

Furthermore, a comparison study of human-associated *E. coli* lineages (ST131, ST95, and ST69) will be conducted between poultry meat *E. coli* and commensal/ clinical human *E. coli* isolates living in Canberra region. Also, a poultry associated lineage (ST117) isolated from both humans and poultry meat sources is selected for further comparison, focusing on VF and plasmid content, resistance profile, and variable gene content, using whole genome sequence data. This comparison study will be carried out to determine if there is transmission of *E. coli* strains between the two hosts, and whether poultry meat serves as a potential risk to humans. The sampling periods for collecting both human and poultry meat *E. coli* isolates will be conducted approximately during the same time frame and in the same area (Canberra region).

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Chapter 2

Factors associated with the presence, genetic diversity, and antimicrobial sensitivity of *Escherichia coli* in poultry samples collected from Canberra, Australia

2.1 Abstract

Escherichia coli contamination is common in retail meat products, especially poultry meat. Antibiotic resistance is rapidly rising in clinically significant bacteria like *E. coli*, hence becoming a global concern. In this study, poultry meat samples (n=306) were collected during the months of November-December 2013, April and August 2014 representing summer, autumn and winter seasons respectively from 16 shops, representing the 3 major supermarket chains in Canberra, Australia and an independent butchery located in each of the four major Canberra town centres. In addition, organic and free-range labelled breast fillet meat samples were also collected. *E. coli* was isolated following enrichment and also by antibiotic selection.

In total, 3415 *E. coli* isolates were recovered. Isolates were assigned to a phylogenetic group using the Clermont quadruplex PCR method and fingerprinted using repetitive element palindromic (REP) PCR using ERIC and CCG primers. A total of 878 *E. coli* isolates were screened for their antimicrobial susceptibility profiles. The probability of detecting *E. coli* and the number of fingerprint types detected per sample, as well as the phylogroup membership of the isolates as well as their antimicrobial sensitivity profile varied, with one of more of retailer, store, meat type, season, and husbandry.

The results of this study demonstrate that poultry meat products are likely to be contaminated with a genetically diverse community of *E. coli* and that the presence of *E. coli* in a sample is not only a consequence of contamination of the meat by the bird's own faecal *E. coli*, but that contamination may occur at multiple points in the production and distribution chain.

2.2 Introduction

Escherichia coli is a common contaminant of the food humans consume, and people may be exposed to viable *E. coli* cells if fresh food products are cooked inadequately or as a result of cross contamination of uncooked food during meal preparation. There are three potentially adverse consequences resulting from the exposure to and subsequent establishment of *E. coli* present in food; disease if a diarrheagenic strain is ingested; the establishment and persistence of a strain capable of causing a subsequent extra-intestinal infection; and as a potential source of mobile genetic elements and antibiotic resistance determinants that might transfer to other strains resident in the host.

Poultry meat is the most consumed meat in Australia, with an annual consumption of 46.2 kilograms per person (http://www.chicken.org.au/chookchat/its-official-chicken-remains-australias-favourite-meat/). *E. coli* is a normal member of the enteric community of poultry and other birds (Blyton et al., 2015) and the vigorous and complex processing of poultry leads to higher levels of bacterial contamination compared with other meat types (Johnson et al., 2005).

E. coli strains capable of causing diarrheal disease are not often encountered in poultry meat (Boyce et al., 1995). However, poultry meat is thought to harbour the most 'human-like' strains of *E. coli*, and therefore may be a potential zoonotic source of extra-intestinal pathogenic strains (ExPEC) (Manges, 2016, Mora et al., 2013, Ewers et al., 2009). Antimicrobials are still widely used in food animal production either for disease prevention or treatment. In Australia, the use of antimicrobials as growth promoters is banned (http://www.chicken.org.au/files/_system/document/acmf_review-judicious_use_of_antimicrobial_agents.pdf). The country is also in a unique position because broad-spectrum antimicrobials like fluoroquinolones, have never been approved for use in food-producing animals (Page, 2011, Cheng et al., 2012). Irrespective of these regulations, the use of veterinary antimicrobials (including food-producing animals) still accounts for two-thirds of the total antimicrobials used (http://www.health.gov.au/internet/main/publishing.nsf/Content/health-pubs-jetacar-cnt.htm/$FILE/jetacar.pdf) in Australia.

The objective of this study was to determine the genetic structure and antimicrobial resistance of *E. coli* isolated from poultry meat products and to investigate the factors contributing variation among meat samples. To this end, a detailed survey of poultry meat products sourced from the Canberra region of Australia was undertaken. Different meat types were sourced from stores of each of the major supermarket chains and an independent butcher located in each of the four major Canberra town centres, and each store was sampled three times over an 8 month period.

2.3 Materials and Methods

2.3.1 *Sampling*

Chicken meat products were sampled from stores of the three major supermarket chains in Australia (Aldi, Coles, and Woolworths) and independent butcheries located in the four main town centres (Belconnen, Gungahlin, Tuggeranong, and Woden) of Canberra, Australian Capital Territory, Australia. Sampling was carried out in late November/early December 2013 (summer), and in April (autumn) and August (winter) 2014. Breast, thigh, and wing meat, as well as mince and whole carcasses from conventionally raised birds were collected from each store. In addition, breast meat labeled as coming from free-range and organically raised birds was also sampled. Packages of each meat type were collected from the display cases in each of the stores belonging to the major supermarket chains. In the case of the independent butcheries, meat samples were not prepackaged, and samples of each meat type were taken from the display case and then packaged individually by the butcher. All meat samples were transported in cooler boxes containing ice packs.

2.3.2 *Isolation of Escherichia coli*

The meat samples were processed within two hours of collection from the retailer. A 20 g portion of the meat from each sample was transferred to a sterile stomacher bag containing 180 ml of maximum recovery diluent enrichment broth (Acumedia, Neogen) and homogenized using a stomacher (BagMixer, Interscience) for 3 minutes. In the case of the whole chicken samples, a 20 g meat sample was taken from the pelvic region

(Supplemental Fig. 2.1). For the whole carcasses collected in August 2013, in addition to taking a 20 g sample of the pelvic region, a 20 g sample from the breast meat was also taken.

The stomacher bags have an internal filter membrane that separates the meat fragments from the homogenate. A subsample of the homogenate was removed from the stomacher bag using a sterile syringe. A 100 µl sample of the homogenate was plated onto a MacConkey agar plate (Acumedia, Neogen) using the spread plate method and the plate was incubated overnight at 35° C. In addition, 1 ml of each homogenate was used to inoculate two flasks containing either 9 ml of lauryl sulfate broth (Acumedia, Neogen) or lysogeny (LB) broth supplemented with vancomycin (20 mg l^{-1}) in order to enrich for *E. coli*. The enrichment cultures were incubated at 35° C for 18 h with shaking at 150 rpm.

Following incubation, a sample from each enrichment culture was dilution streaked onto a MacConkey agar plate. A 100 µl aliquot of each enrichment culture was spread onto a MacConkey agar plate and disks (Becton, Dickinson and Company) containing one of 8 antimicrobials [amoxicillin-clavulanic acid (20/10 µg), nalidixic acid (30 µg), nitrofurantoin (100 µg), gentamicin (10 µg), ampicillin (10 µg), trimethoprim-sulfamethoxazole (23.75/1.25 µg), ceftiofur (30 µg), and tetracycline (30 µg)] were placed onto the plate. The agar plates were then incubated overnight at 35° C.

Following incubation a single putative *E. coli* colony (pink, non-mucoid) was selected from each of the MacConkey plates used for a meat sample. In the case of the plates containing the antimicrobial disks, any putative *E. coli* colony growing within the zone of inhibition of any antimicrobial disk was also selected for further characterization.

Putative *E. coli* isolates were sub-cultured onto Simmons citrate agar and urease agar (Acumedia, Neogen) and incubated overnight for 24 to 48 hours at 35° C. Any isolate found to be lactose positive and citrate and urease negative were considered to be *E.*

coli. Freezer cultures of all *E. coli* isolates were made by adding 1 ml of an overnight lysogeny (LB) broth culture to 50 μl of glycerol and these were stored at -80 ^{o}C.

2.3.3 *Molecular characterization*

DNA extraction was performed using DNAzol (Molecular Research Center Inc.) and a 200 μl aliquot of an overnight lysogeny broth culture according to the manufacturer's protocol. All isolates were assigned to a phylogroup by performing the Clermont quadruplex PCR assay (Clermont et al., 2013). In addition, the identity of the isolate as *E. coli* was confirmed using a PCR assay for the *E. coli* specific genes *iudA* and *gadA/B* (McDaniels et al., 1996).

REP-PCR (DNA Fingerprinting) was performed using ERIC (Versalovic et al., 1991) and CCG (Adamus-Bialek et al., 2009) primers on all *E. coli* in order to identify the different REP-types present within a meat sample. ERIC and CGG primers-based PCR were performed in an Applied Biosystems 2720 Thermal Cycler. The primers used for ERIC fingerprinting were ERIC1 and ERIC2 and $N_6(CGG)_4$ for CGG. PCR reactions (20 μl) for ERIC contained 10 ng of DNA template, 4 μl of 5x MyTaq Red Reaction buffer (Bioline), primers at 0.8 μM each and 1.0 U MyTaq DNA polymerase (Bioline). PCR reaction for CGG (also 20 μl) also contained 10 ng of DNA template, 1x PCR buffer (Fisher Biotech), $MgCl_2$ (Bioline) at 3.5 mM, primer at 0.8 μM and 1.0 U MyTaq DNA polymerase.

All isolates for each meat sample were characterized using the ERIC and CCG primers. The REP-PCR products from all isolates from a single meat sample were run together on an agarose gel and the PCR products were loaded based on the isolate's phylogroup membership. All isolates with a unique ERIC and CCG fingerprint from a meat sample were then compared among all meat samples from the same shop and all unique REP types found in all of the samples from a shop were then compared among shops. REP-types were not compared among seasons.

All subsequent characterization and statistical analyses were restricted to a single example of each REP type identified in a meat sample.

2.3.4 *Antimicrobial susceptibility testing*

Antimicrobial susceptibility test was performed for all the unique *E. coli* REP-types found within a meat sample using European Committee on Antimicrobial Susceptibility Testing (EUCAST) disk diffusion method (version 3.0; EUCAST [http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Disk_test_documents/Manual_v_3.0_EUCAST_Disk_Test.pdf]) on Mueller-Hinton agar plates (Acumedia, Neogen) using 13 clinically relevant antimicrobials, namely, amoxicillin-clavulanic acid (20/10 µg), ampicillin (10 µg), nalidixic acid (30 µg), nitrofurantoin (100 µg), gentamicin (10 µg), trimethoprim-sulfamethoxazole (23.75/1.25 µg), tetracycline (30 µg), ciprofloxacin (5 µg), cefazolin (30 µg), cefotaxime (30 µg), ceftazidime (30 µg), ceftiofur (30 µg) and ertapenem (10 µg) (Becton, Dickinson and Company). The zone of inhibition diameters were measured using the instrument ProtoCol 3 (Synbiosis). The *E. coli* strains were classified as susceptible, intermediate or resistant to an antimicrobial, based on their zone diameters after 18 to 24 hours incubation at 35^{o}C using the EUCAST breakpoints.

2.3.5 *Statistical analyses*

The main approaches used to statistically analyse the data are presented below. Further details of the analyses and the results of all analyses are presented in the Supplemental Results.

2.3.5.1 Presence of *E. coli* and number of REP-types in a sample. To determine which factors influenced whether or not *E. coli* was detected in a meat sample, we fitted generalised linear models (family = binominal, 1 = presence, 0 = absent) using the statistical package in R (http://www.R-project.org/). Additionally, to determine which factors influenced the number of *E. coli* REP types detected in a meat sample, we also fitted generalised linear models of the family quasi-poisson using the stats package in R

(http://www.R-project.org/). The response variable in the richness analyses was the number of unique REP types detected in a meat sample.

2.3.5.2 Distribution of phylogenetic groups (phylogroups). To determine which factors influenced the phylogroups detected in the meat samples that contained *E. coli*, we fitted generalised linear regression models (family = binomial) using the stats package in R (http://www.R-project.org/). It was assumed that the different strains of *E. coli* in a sample did not compete, as it was unlikely that the strains were growing in the samples given they are stored at 5°C. Further, *E. coli* cell densities in these meat samples were also low, as *E. coli* was seldom detected in a homogenate of a meat sample prior to the sample's enrichment. Finally, as an average of 19 isolates were obtained from each sample, there was the potential for all *E. coli* phylogroups to be detected in a sample. For these reasons, the detection of one phylogroup in a sample was considered independent of the detection of the other phylogroups, and thus, the different phylogroups were analysed in separate analyses. The response variables in the analyses were whether or not the phylogroup in question was detected in a sample that had *E. coli*. The REP type richness of the samples was included as a covariate as it was expected that the probability of detecting a phylogroup would increase as the number of strains detected increased.

2.3.5.3 Antimicrobial resistance. Isolates that were classified as intermediate according to their inhibition zone diameters were generally grouped with the resistant isolates for analysis. However, isolates classified as intermediate to nitrofurantoin, ampicillin, or cefazolin were grouped with the susceptible isolates following Blyton et al. (2015).

Resistance is often correlated among different antibiotics that either belong to the same class or because the genes that encode resistance are co-located on the same integron gene cassettes. Therefore, we performed a multiple correspondence analysis in TANAGRA (Rakotomalala, 2005) to identify and summarise any associations between the insistences of resistance to different antibiotics. Only antibiotics for which resistance was observed in greater than 5% of REP-types isolates were included in the

analysis. Significant contributors to each axis were identified using v-tests with the standard critical cut off of 4.

To investigate which factors influenced the incidence of antibiotic resistance among the *E. coli* isolates we fitted a series of generalised linear regression models (family = binomial) using the stats package in R (R-Core-Team 2012). The response variables in the analyses were whether or not a particular unique REP type (within a sample) was resistant to one or more of a group of antibiotics. Antibiotics that clustered together in the multiple correspondence analysis were grouped together. The phylogroups of the isolates were included as a covariate as antibiotic resistance is known to vary between the phylogroups (Skurnik et al., 2005, Johnson et al., 2009, Blyton et al., 2015).

2.4 Results

2.4.1 Presence of *E. coli*

Across all 306 meat samples tested *E. coli* was detected in 77.5% (237) of samples. For the conventional meat samples there was a significant effect of meat type and retailer on the probability of detecting *E. coli* in a sample (Supplemental Table 2.1). Meat samples from conventionally raised birds sourced from the independent butchers were significantly less likely to have *E. coli* than meat samples from any of the major retailers (Butchers vs Woolworths, Coles or ALDI: $p < 0.001$) (Fig. 2.1a). Additionally, and regardless of retailer, *E. coli* was significantly more likely to be detected in meat from conventionally raised birds taken from the pelvic region of whole carcasses than from mince, breast meat or wings (pelvic vs mince: $p = 0.019$, vs breast meat: $p = 0.013$, vs wings: $p = 0.015$). There was also a borderline non-significant effect of season on the presence/absence of *E. coli* in a conventional meat sample. The probability of detecting *E. coli* in a sample was significantly higher in summer than in autumn ($p = 0.036$), while the probability of detection in winter was intermediate.

For the set of samples collected during the winter, breast meat from the carcass of whole chickens raised conventionally was also sampled in addition to the other meat

types. *E. coli* was significantly less likely to be detected in breast meat from whole carcasses than in any other meat type (whole carcass breast meat vs pelvic: p = 0.004; vs thigh: p = 0.004, vs mince: p = 0.019, vs breast: p = 0.020, vs wings: p = 0.038). *E. coli* was not detected in any of the whole breast meat samples collected from ALDI.

We determined whether the rearing method influenced whether or not *E. coli* was detected in a breast meat sample. Only samples from Coles and Woolworths were included in these analyses. The rearing method was found to significantly affect the probability of detecting *E. coli* in breast meat samples (p < 0.003). Both organic and conventional breast meat were significantly more likely to have *E. coli* than free-range breast meat (free range vs organic: p = 0.005; vs conventional: p = 0.011), while there was no significant difference between organic meat and conventional meat (p = 0.491).

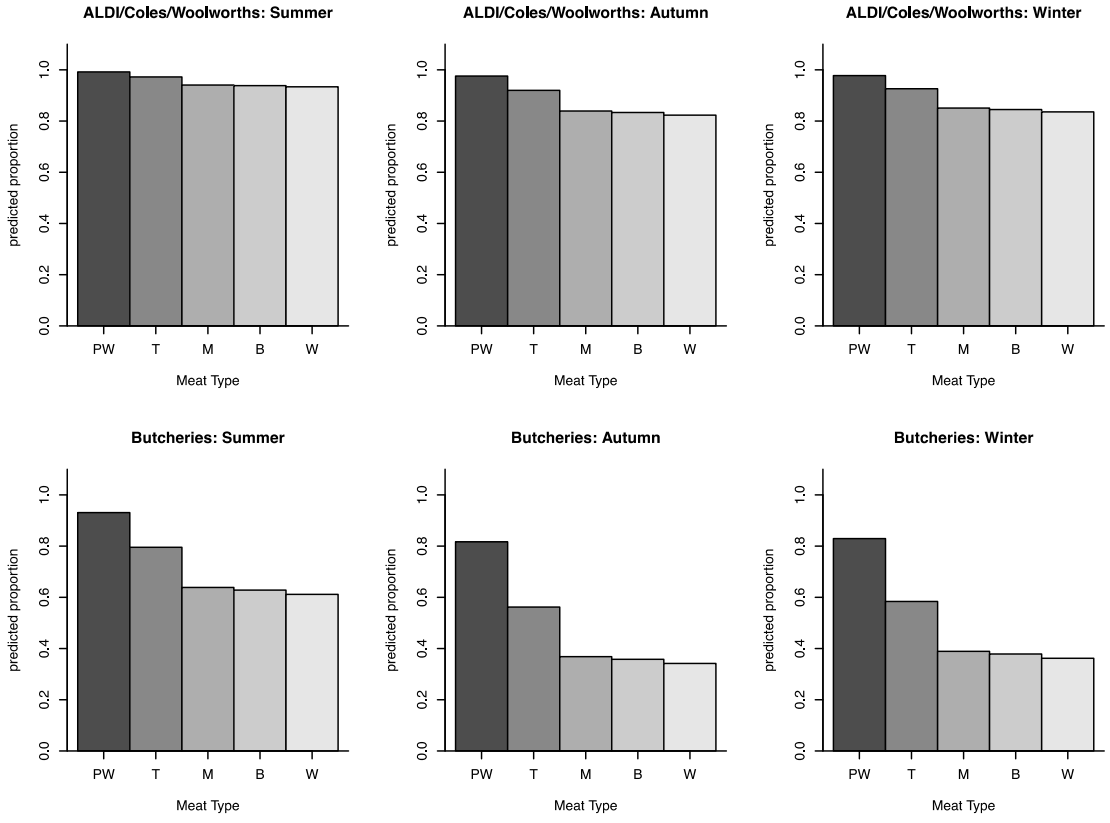

Figure 2.1a: Predicted probability of detecting *E. coli* in a meat sample with respect to season, meat type, and retailer. M = mincemeat, T = thigh meat, W = wings, B = breast meat and PW = samples taken from the pelvic region of whole carcasses.

2.4.2 Number of REP-types

An average of 2.3 *E. coli* REP-types (range, 0 – 9) were detected per meat sample. There was no significant effect of town-centre on *E. coli* richness (number of REP-types) in meat samples from conventionally raised birds (Supplemental Table 2.2). Meat type had a significant effect on the number of strains detected in a sample, however, the effect of meat type depended on the retailer and season that the sample was collected from (Supplemental Table 2.2). Among the samples collected from the independent butcheries, across all seasons, samples taken from the pelvic region of whole carcasses had significantly higher richness than any of the other meat types, while the other conventional meat types had very low richness compared to the samples from the other retailers (Fig. 2.1b). Among the samples collected from ALDI, there were no significant differences in *E. coli* richness between the meat types in summer or autumn, but in winter, thigh and breast meat had significantly higher richness than mince (Fig. 2.1b). There was no significant difference between Coles and Woolworths in the effect of meat type on REP-type richness ($p = 0.2736$). Among the meat samples from conventionally raised birds collected from Woolworths and Coles, the mince and wing meat had significantly higher richness than breast meat in summer, while there were no significant differences between the meat types in autumn (Fig. 2.1b). In winter, mince and thigh meat from Coles and Woolworths had significantly higher richness than wing or breast meat.

Comparing *E. coli* REP-type richness in samples taken from the breast meat from whole carcasses of conventionally raised birds to that of other conventional meat samples collected in winter, we found that a relatively low number of REP types were detected in the whole breast meat. Breast meat samples taken from whole carcasses sourced from Coles and Woolworths had significantly lower richness than thigh meat, mince and the pelvic region samples (Fig. 2.1b). Additionally, the breast meat from whole carcasses sourced from the butcheries had a similar level of richness to the other meat samples from the butcheries, but which was considerably lower than the pelvic region samples (Fig. 2.1b).

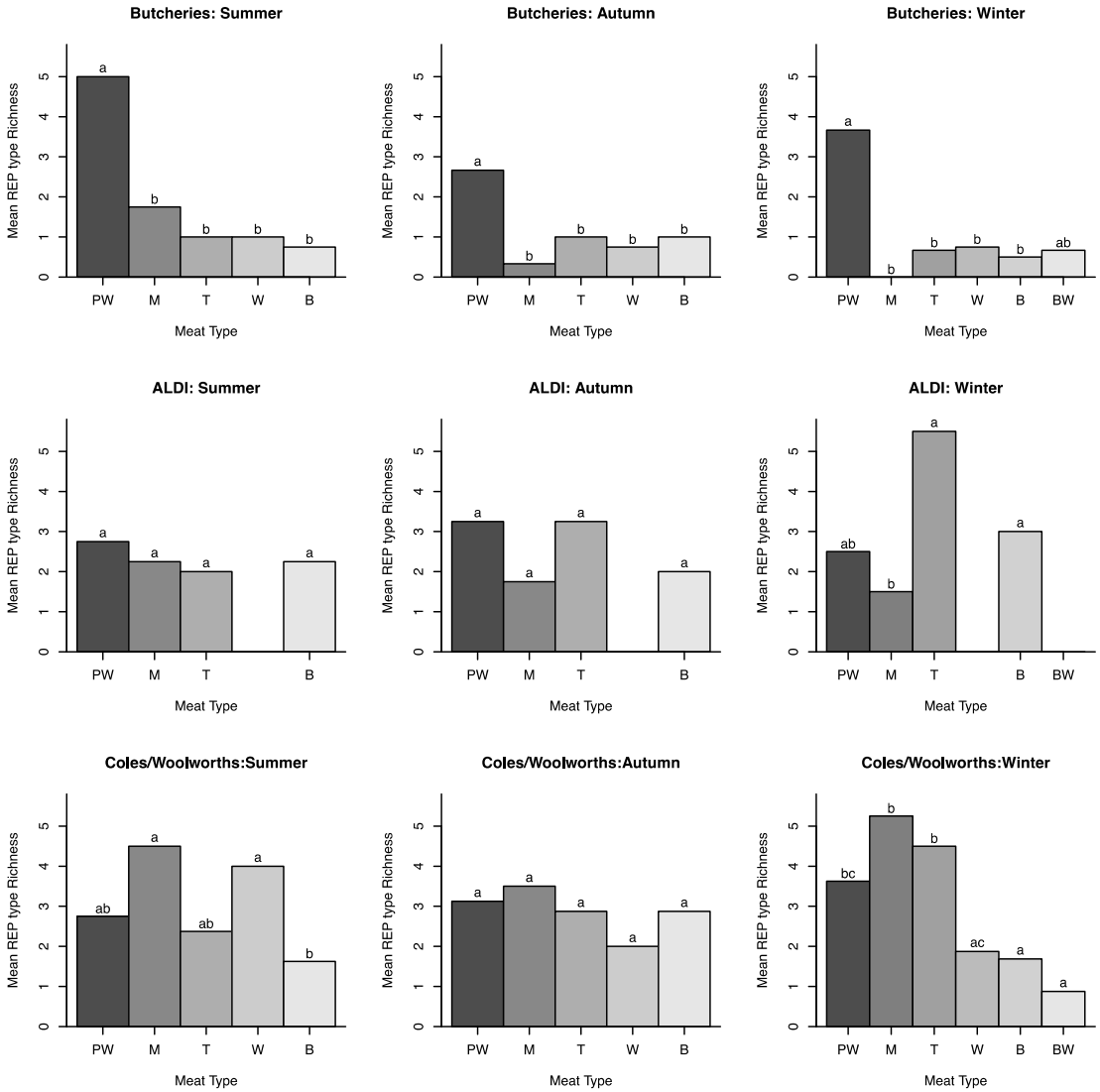

Figure 2.1b: Mean number of *E. coli* REP types in the conventional meat samples. PW = samples taken from the pelvic region of whole carcasses, T = thigh meat, M = mincemeat, B = breast meat, W = wings and BW = samples taken from the breast of whole carcasses. Meat types with the same letter were not significantly different from each other (p>0.05).

The rearing method was found to significantly affect the REP-type richness detected in the breast meat samples ($p < 0.001$). Breast meat from organically and conventionally raised birds were found to have significantly more *E. coli* REP-types than breast meat from free-range birds (Fig. 2.2). The analysis that investigated which factors influenced richness among breast meat samples from organically reared birds revealed that samples collected from Coles had significantly higher richness than those from Woolworths ($p = 0.021$). However, neither town-centre nor season explained any of the variation in richness between the breast meat samples from organically raised birds (Supplemental Table 2.3). By contrast, town-centre was found to explain a significant amount of the variance in the number of *E. coli* REP-types recovered from breast meat

samples from free-range birds (Supplemental Table 2.4). Samples from Woden had significantly higher richness than samples from Tuggeranong (p = 0.028), while there were no other significant differences between the town-centres. Neither retailer nor season significantly explained the variation in richness between the free-range meat samples (Supplemental Table 2.4).

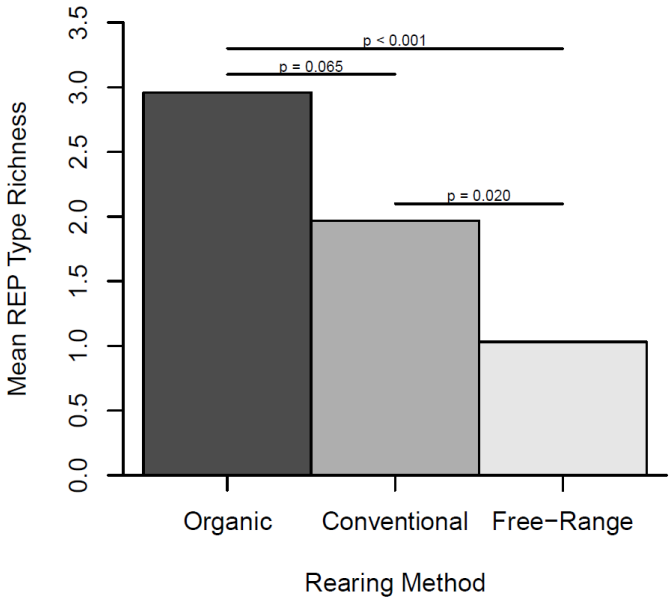

Figure 2.2: Mean number of *E. coli* REP types in breast meat samples produced using different rearing methods.

2.4.3 Phylogenetic group (phylogroup) distribution

A total of 724 *E. coli* isolates with unique REP-type profiles within a meat sample were detected in the 237 samples that were positive for *E. coli.* Among these, 27.2% (197) were assigned to phylogroup A, 21.3% (154) were phylogroup B1, 8.3% (60) were B2, 10.6% (77) were D, 16.4% (119) had an E Clermont profile and 15.2% (110) were assigned to phylogroup F. The remaining seven isolates had Clermont profiles that were not consistent with any of the phylogroups.

As expected, the probability of detecting each of the phylogroups in a meat sample from a conventionally raised bird sourced from the major retailers significantly increased as REP-type richness increased (Supplemental Tables 2.5-2.10). There was a significant effect of meat type on the probabilities of detecting B1 and B2 strains in meat samples from the major retailers (Supplemental Tables 2.5-2.6). B1 strains were significantly less

likely to be detected in mince samples than in samples of any of the other meat types (Fig. 2.3). By contrast, B2 strains were least likely to be detected in pelvic region sample from whole carcasses and most likely to be detected in mince, while the frequencies of B2 strains were intermediate in the other meat types (Fig. 2.4). The probability of detecting an A strain in a meat sample from conventionally raised birds was independent of meat type (Supplemental Table 2.7). However, the probability of detecting an A strain varied with store, but not with retailer or town centre (Supplemental Table 2.7; Fig. 2.5). The probability of detecting a D strain was only predicted by REP-type richness (Supplemental Table 2.8). There was a significant season effect on the probability of detecting an E strain in a sample (Supplemental Table 2.9), with the chance of detecting an E strain significantly higher in summer than in winter (Fig. 2.6). The chance of detecting an F strain appeared to be idiosyncratic with significant interaction effects between meat type and season, retailer and season as well as between town-centre and season (Supplemental Table 2.10; Fig. 2.7).

The rearing method did not have a significant effect on the probability of detecting any of the phylogroups in the breast meat samples (A: $p = 0.914$, B1: $p = 0.684$, B2: $p = 0.734$, D: $p = 0.191$, E: $p = 0.957$, F: $p = 0.117$). Within the organic and free-range breast meat samples, only the probability of detecting D strains could be explained by any of the explanatory variables tested (Supplemental Tables 2.11- 2.16). D strains were significantly more likely to be detected in breast meat samples collected in winter than in summer ($p = 0.029$), while D strains were not detected in organic or free-range meat collected in autumn. D strains were also significantly more likely to be detected in samples from Woolworths than from Coles ($p = 0.034$).

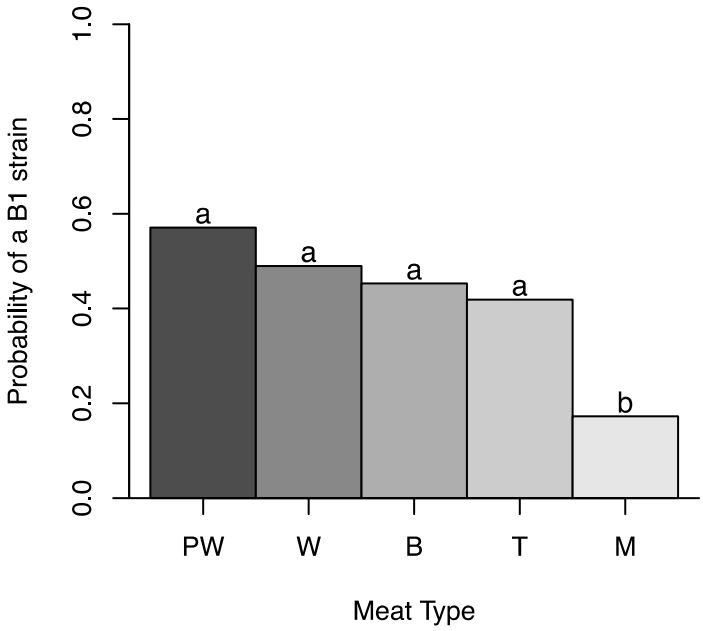

Figure 2.3: Probability that a phylogroup B1 strain was detected in a conventional meat sample from the major retailers, as predicted from the final generalised linear model when the REP type richness was set to 3. PW = samples taken from the pelvic region of whole carcasses, M = mincemeat, T = thigh meat, W = wings, B = breast meat. Meat types with the same letter were not significantly different from each other (p>0.05).

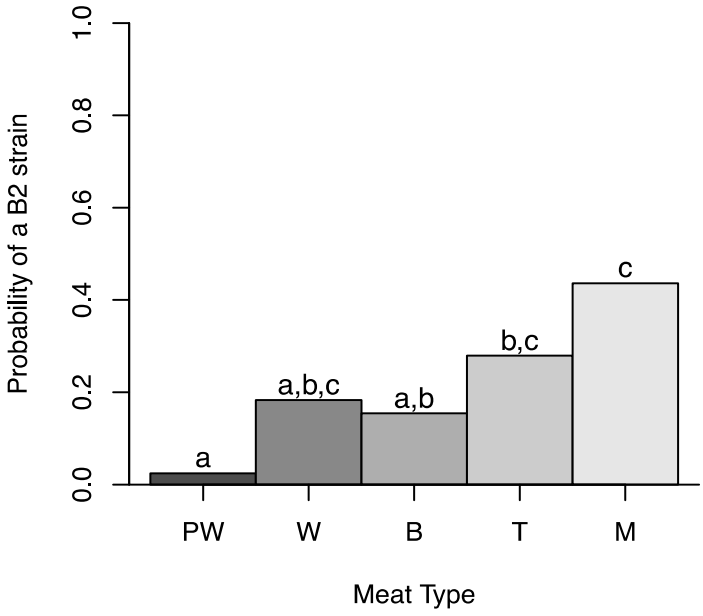

Figure 2.4: Probability that a phylogroup B2 strain was detected in a conventional meat sample from the major retailers, as predicted from the final generalised linear model when the REP type richness was set to 3. PW = samples taken from the pelvic region of whole carcasses, M = mincemeat, T = thigh meat, W = wings, B = breast meat. Meat types with the same letter were not significantly different from each other (p>0.05).

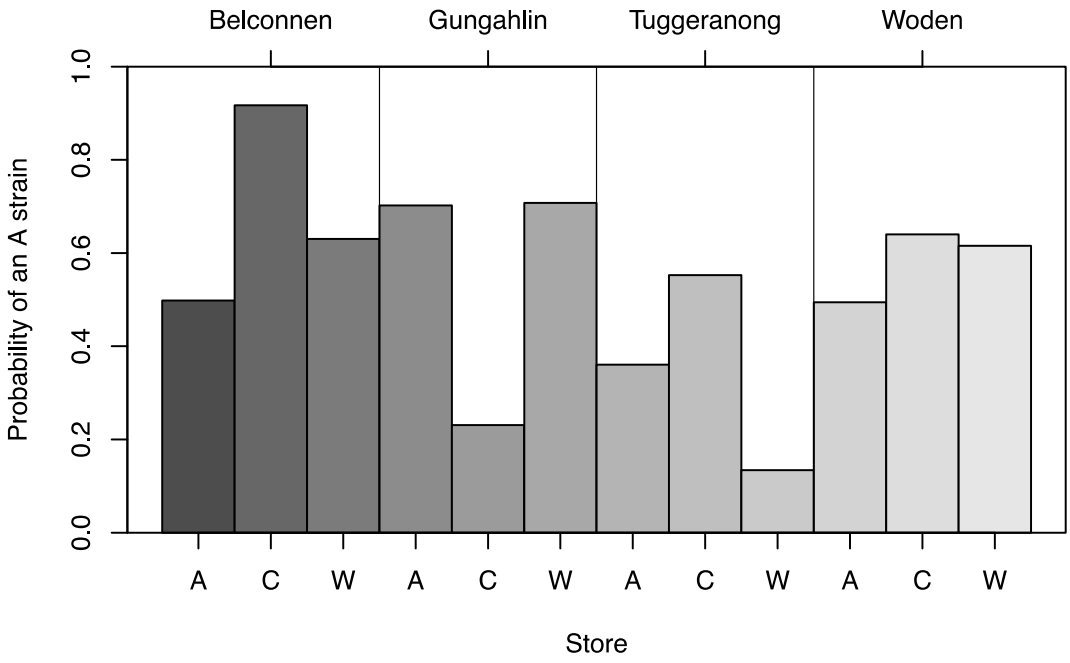

Figure 2.5: Probability that a phylogroup A strain was detected in a conventional meat sample from the major retailers by store, as predicted from the final generalised linear model when the REP type richness was set to 3. A = ALDI, C = Coles, W = Woolworths. Mince samples were not included in the final generalised linear model.

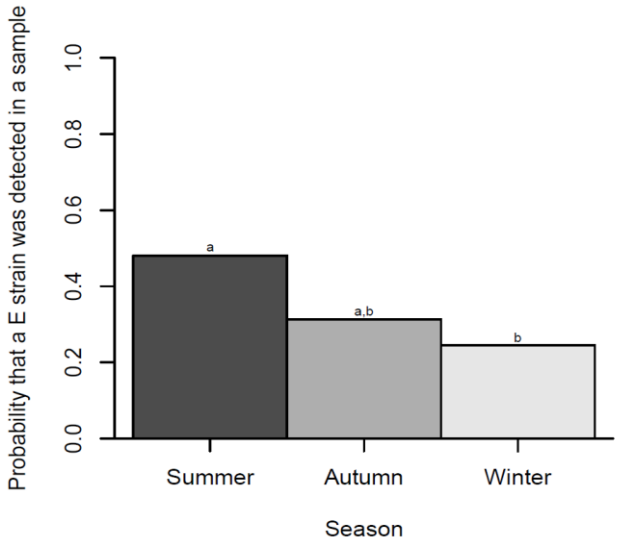

Figure 2.6: Probability that a phylogroup E strain was detected in a conventional meat sample from the major retailers by season, as predicted from the final generalised linear model when the REP type richness was set to 3. Seasons with the same letter were not significantly different from each other ($p>0.05$).

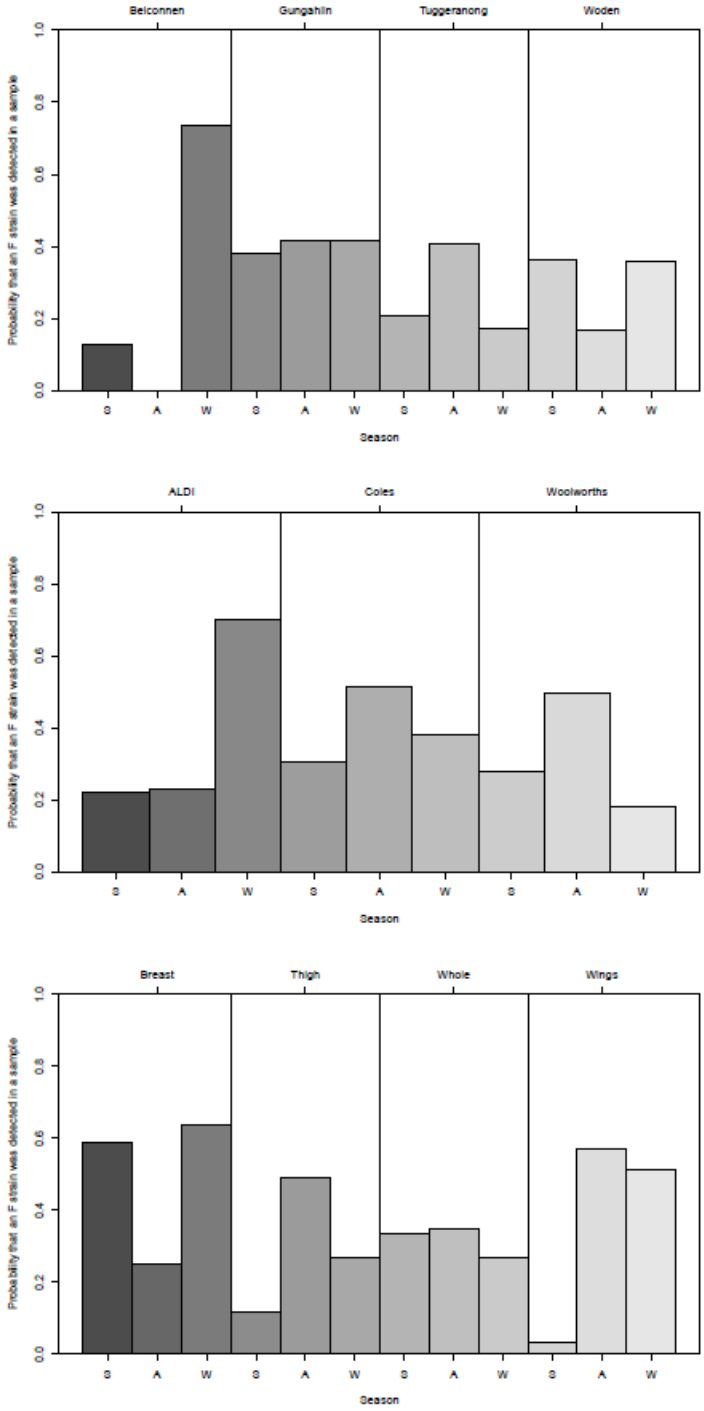

Figure 2.7: Probability that a phylogroup F strain was detected in a conventional meat sample from the major retailers by season and town centre (top), retailer (middle) and meat type (bottom). Values were predicted from the final generalised linear model when the REP type richness was set to 3. F strains were not detected in Belconnen during autumn.

2.4.4 Antimicrobial resistance

Resistance was observed for 11 tested antimicrobials (Table 2.1). Resistance to tetracycline was common (39.0%), to ampicillin (27.4%), and to

trimethoprim/sulfamethoxazole (16.8%). Resistance to cefazolin and to amoxicillin/clavulanic acid exceeded 5% but were comparatively uncommon. Resistance to the remaining six antibiotics were found in less than 5% of REP types and these antibiotics were not included in further analyses.

Table 2.1. Antibiotic resistance status of 724 *E. coli* isolated from poultry meat

Antimicrobial	Class	Number of resistant isolates (%)
Gentamicin	aminoglycoside	21 (2.7%)
Cefazolin	1st generation cephalosporin	59 (7.7%)
Ceftazidime	3rd generation cephalosporin	7 (0.9%)
Cefotaxime	3rd generation cephalosporin	5 (0.7%)
Ceftiofur	3rd generation cephalosporin	0 (0.0%)
Ertapenem	carbapenem	0 (0.0%)
Ciprofloxacin	fluoroquinolone	14 (1.8%)
Trimethoprim/sulfamethoxazole	folate pathway inhibitor	129 (16.8%)
Nitrofurantoin	nitrofuran	14 (1.8%)
Ampicillin	Penicillin	210 (27.4%)
Tetracycline	tetracycline (polyketide)	299 (39.0%)
Nalidixic acid	quinolone	37 (4.8%)
Amoxicillin/clavulanic acid	β-lactam & β-lactamase inhibitor	58 (7.6%)

The first and second axes of the correspondence analysis explained 34.3% and 31.4% of the variation in antibiotic resistance, respectively (Fig. 2.8). Resistance to two antibiotics (amoxicillin/clavulanic acid and cefazolin) were significant contributors to Axis 1 and were grouped for the regression analyses. Resistance to three antibiotics (ampicillin, trimethoprim/sulfamethoxazole, and tetracycline) were significant contributors to Axis 2 (Fig. 2.9). These three antibiotics were grouped for the regression analyses.

The phylogroup of the *E. coli* isolates from conventional meat samples from conventionally raised birds did not explain a statistically significant amount of the variation in the frequency of resistance to either of the two groups of antibiotics, but was retained as a covariate in the analyses (Supplemental Tables 2.17 & 2.18). A statistically significant amount of the variation in the frequency of resistance to one or both of amoxicillin/clavulanic acid and cefazolin was explained by meat type (Supplemental Table 2.17). Resistance to either amoxicillin/clavulanic acid and/or cefazolin was detected significantly more often among isolates from mince than among isolates from breast or thigh meat (Fig. 2.8). The frequency of resistance to one or more of ampicillin, trimethoprim/sulfamethoxazole and tetracycline varied significantly among stores (Supplemental Table 2.18) and the frequency of resistance to these antibiotics was lower at the Gungahlin and Tuggeranong butcheries than at the other stores (Fig. 2.9). When the butcheries were removed from the analysis, store no longer explained a significant amount of the variation in the frequency of resistance to one or more of ampicillin, trimethoprim/sulfamethoxazole and tetracycline ($p = 0.07$). Nalidixic acid resistance occurred more often among *E. coli* isolates sampled from the pelvic region of whole carcasses and mincemeat than among isolates from wing, thigh or breast meat (Fig. 2.10). The incidence of nalidixic acid resistance was also significantly explained by season. Nalidixic acid resistance occurred significantly more often among isolates from summer than winter, with the incidence intermediate in autumn (Fig. 2.11). Overall rearing method explained a significant amount of the variation in frequency of antibiotic resistance *E. coli* in breast meat samples. In the rearing method analyses, the phylogroup membership of the strain explained a significant amount of the variation in the frequency of resistance to one or more of the antimicrobials ampicillin, trimethoprim/sulfamethoxazole and tetracycline ($p = 0.01$). The rearing

method significantly explained the incidence of antibiotic resistance in the case of amoxicillin/clavulanic acid and cefazolin as well as for ampicillin, trimethoprim/sulfamethoxazole and tetracycline after taking into account any effect of the phylogroups (rearing method: CZ-AMC, p =0.01; AM-SXT-TE, p = 0.01). Resistance to amoxicillin/clavulanic acid and/or cefazolin occurred significantly more often among isolates sampled from organic and free-range breast meat than among isolates from conventional meat (Fig. 2.12). By contrast, the incidence of resistance to one or more of ampicillin, trimethoprim/sulfamethoxazole and tetracycline was significantly higher among isolates sampled from conventional breast meat than among isolates from free range or organic meat (Fig. 2.13).

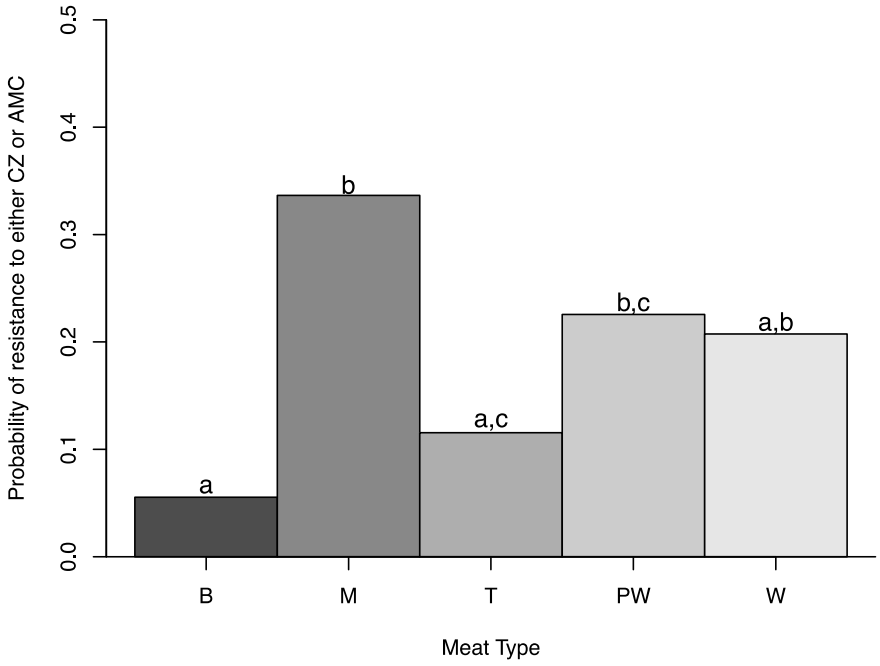

Figure 2.8: Probability that an *E. coli* strain isolated from conventional meat was resistant to either amoxicillin/clavulanic acid and/or cefazolin by meat type. Values were predicted from the final generalised linear model with the proportions of the different phylogroups within each meat type set to the mean proportions across all samples. PW = samples taken from the pelvic region of whole carcasses, M = mincemeat, T = thigh meat, W = wings, B = breast meat. Meat types with the same letter were not significantly different from each other (p>0.05).

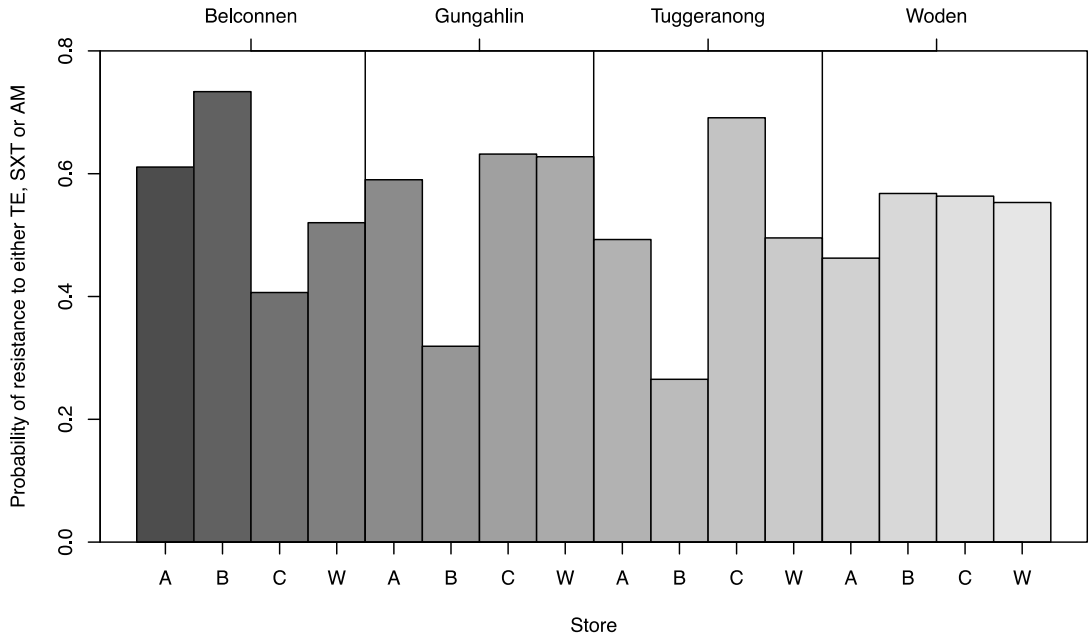

Figure 2.9: Probability that an *E. coli* strain isolated from conventional meat was resistant to one or more of ampicillin, trimethoprim/sulfamethoxazole and tetracycline by store. Values were predicted from the final generalised linear model with the proportions of the different phylogroups within each store set to the mean proportions across all samples. A = ALDI, C = Coles, W = Woolworths.

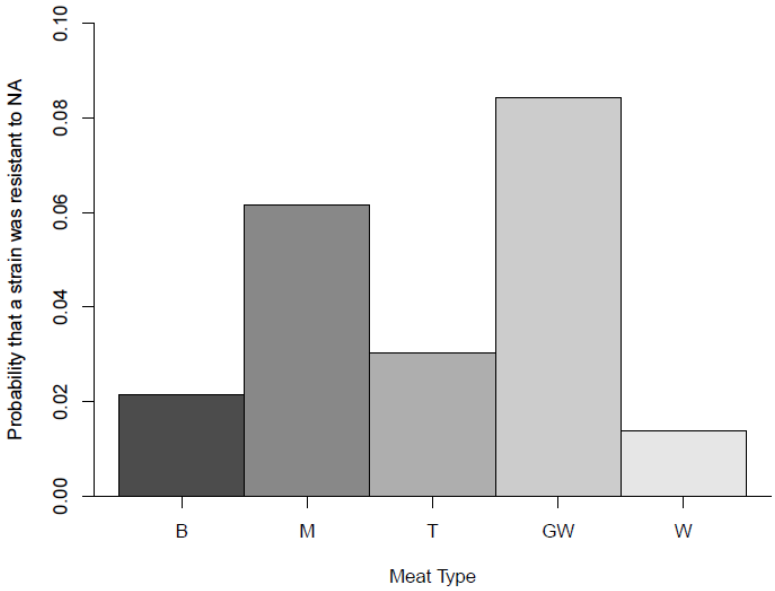

Figure 2.10: Probability that an *E. coli* strain isolated from conventional meat was resistant to nalidixic acid by meat type. Values were predicted from the final generalised linear model with the proportions of the different phylogroups within each meat type set to the mean proportions across all samples. B2 isolates were not included in the analysis. PW = samples taken from the pelvic region of whole carcasses, M = mincemeat, T = thigh meat, W = wings, B = breast meat.

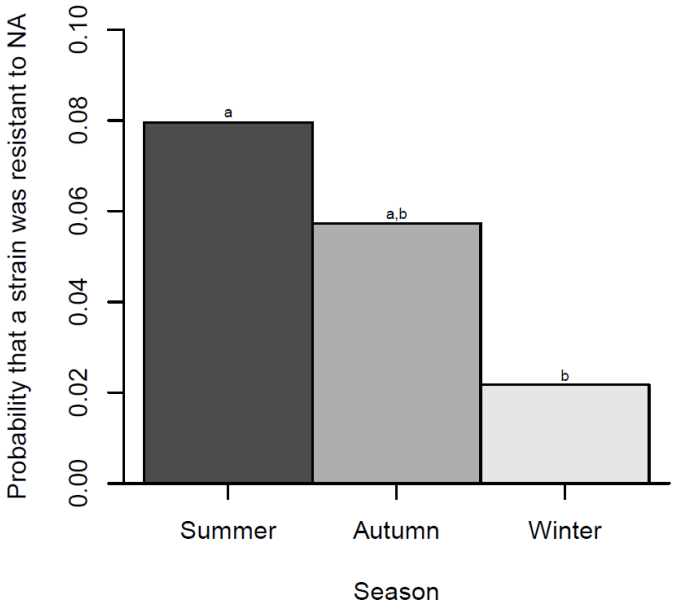

Figure 2.11: Probability that an *E. coli* strain isolated from conventional meat was resistant to nalidixic acid by season. Values were predicted from the final generalised linear model with the proportions of the different phylogroups within each season set to the mean proportions across all samples. B2 isolates were not included in the analysis.

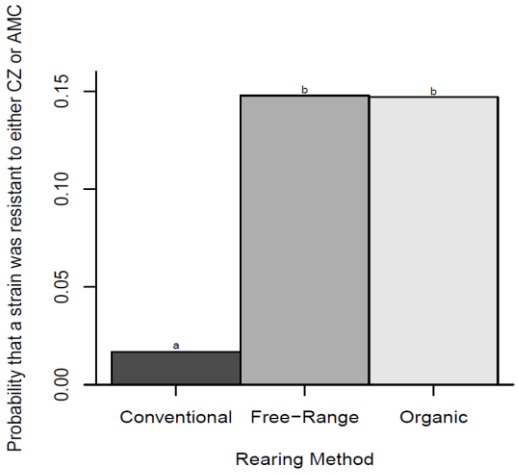

Figure 2.12: Probability that an *E. coli* strain was resistant to either amoxicillin/clavulanic acid and/or cefazolin by rearing method. Values were predicted from the final generalised linear model with the proportions of the different phylogroups within each meat type set to the mean proportions across all samples. Phylogroup D isolates were not included in the analysis as neither amoxicillin/clavulanic acid nor cefazolin resistance were detected in those isolates. Rearing methods with the same letter were not significantly different from each other ($p>0.05$).

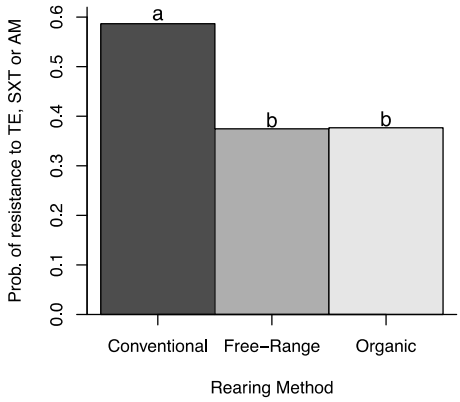

Figure 2.13: Probability that an *E. coli* strain was resistant to one or more of ampicillin, trimethoprim/sulfamethoxazole and tetracycline by rearing method. Values were predicted from the final generalised linear model with the proportions of the different phylogroups within each meat type set to the mean proportions across all samples. Rearing methods with the same letter were not significantly different from each other ($p>0.05$).

2.5 Discussion

Placing the results of this study in context requires a brief description of the poultry industry and poultry meat production in Australia (http://www.chicken.org.au/index.php). Chicken meat production is predominately vertically integrated, where meat production companies own the breeding farms, hatcheries, feed mills, some broiler growing farms, and the processing plants. About 800 growers produce about 80% of meat chickens under contract to the production companies. Two companies are responsible for about 70% of the chicken meat production in Australia: Baiada Poultry and Inghams Enterprises. There are an additional six medium-sized companies that supply between 3% and 9% of the national market, and a large number of smaller processors (ACMF, when accessed). In the present study, the producer(s) of the meat samples obtained from the independent butcheries were unknown. All of the meat samples from conventionally raised birds obtained from the major retailers, excepting mince from Aldi, were from just one of the major producers.

About 600 million chickens are slaughtered per year in Australia or about 1.65 million per day (statement of fact, ACMF). A typical production farm houses about 320,000 chickens in 8 sheds of same ages (ACMF). Once the chicks arrive at a production farm

the first harvest will occur as early as 30-35 days and the last at 55-60 days (ACMF). A production farm typically grows about 5-6 batches of chicken per shed per year (ACMF). Consequently, if expressed on a daily basis, a typical production farm produces about 5,000 chickens per day (ACMF). Australia's largest processing plant, kills and processes about 90,000 birds per day, a number equivalent to the daily production of about 20 typical production farms.

Husbandry practices differ for birds raised conventionally, as free range, or as certified organic (ACMF). Conventionally raised chickens are barn-raised with no access to outdoor forage areas; stocking densities are 28-40 kgm^{-2} and are harvested at 35-55 days of age; birds may be given antibiotics for prophylactic and/or therapeutic purposes (ACMF). Free-ranged chickens are barn-raised with access to outdoor forage areas; stocking densities are 16-34 kgm^{-2} and are harvested at 35-55 days of age; birds may or may not be given antibiotics (depends on accreditation program) for prophylactic and/or therapeutic purposes (ACMF). Organically raised chickens are barn-raised with no access to outdoor forage areas; stocking densities are 25 kgm^{-2} and are harvested at 65-80 days of age; birds may be given antibiotics for prophylactic and/or therapeutic purposes but cannot then be sold as organic (ACMF).

The samples collected at different times of the year undoubtedly represent different production batches of chickens. Excepting mince sourced from Aldi, all meat samples from conventionally raised birds were from a single major producer. Consequently, it is not known if the meat samples from conventionally raised birds sourced from different stores of the same retailer or from different retailers could represent birds from the same production batch. Given the numbers involved, it seems very unlikely that breast, thigh, or wing meat samples represent portions of the same bird.

E. coli was most likely to be detected in pelvic region samples compared to all other meat types and breast meat samples from whole carcasses were least likely to harbour *E. coli*. Meat from conventionally raised birds and sourced from the major retailers were more likely to harbour *E. coli* than meat samples from the independent butcheries. Most

likely, the pelvic region of whole carcasses are more likely to be exposed to bacterial contamination than the breast portion of the same, and consequently it is more likely that they may become contaminated. In most Australian processing plants, carcasses are washed and cooled in a water and ice mixture and this water is sanitised through the addition of chlorine. This process likely explains the low level of *E. coli* detected in breast meat taken from whole carcasses relative to the other cuts of meat.

Why breast meat taken from free range birds should be significantly less likely to harbour *E. coli* as compared to breast meat from organically or conventionally raised birds is unknown. Husbandry differences are greatest between organically and conventionally raised birds, yet the probability of detecting *E. coli* is similar. It is not known if conventionally and organically raised birds are processed in the same plants while free-range birds are processed in different plants.

The number of *E. coli* genotypes (REP-types) varied with retailer, meat type, and season. Processing undoubtedly plays a role in terming the number of strains (REP-types) detected in a meat sample although some of the observed outcomes appear to be counter-intuitive. Why, for example do most meat types from independent butchers have lower *E. coli* diversity, when these meat types are typically displayed together in open cabinets. It could be assumed that samples representing a portion of the bird (e.g. thigh meat) have likely been handled more frequently and therefore more likely to be contaminated during processing. Similarly, a mince sample represents meat from multiple birds and it could therefore be assumed that more strains would be present than would be the case for samples sourced from a single bird. However, mince samples do not consistently harbour more strains than other meat types. Neither is it obvious why the number of strains detected in different meat types differs with the time of year the sample was collected (Fig. 2.1b). For samples collected from Coles and Woolworths in winter, mince and thigh meat samples had the greatest diversity compared to other meat types, in summer it was mince and wing meat samples with the greatest diversity, while in autumn there was no difference in the numbers of strains detected among meat types.

The probability of detecting *E. coli* in a sample of breast meat varied with rearing method, and these differences were reflected in the number of strains detected per breast meat sample, with the greatest number of strains found in chickens raised organically and the fewest strains in breast meat samples from free range chickens.

On average, across similar studies phylogroup A strains have represented 36% of the *E. coli* recovered from the faeces of commercial meat chickens, 23% were B1 strains, 17% B2, and 24% were D strains (Johnson et al., 2006, Ewers et al., 2009, Jakobsen et al., 2010, Obeng et al., 2012). A very similar distribution has previously been observed for isolates from retail chicken meat: 37% phylogroup A, 26% B1, 10% B2 and 28% D (Johnson et al., 2006, Jakobsen et al., 2010, Kluytmans et al., 2013). Compared to previous studies, in the present study phylogroup A strains were less common (27%) and 'D' strains more common (43%), the relative frequency of B1 and B2 strains were similar to previous estimates.

The probability of detecting strains belonging phylogroup A, the single most common phylogroup, in a meat sample varied with the retailer (Aldi, Coles, Woolworths) and the town centre in which the store was located. For example, a phylogroup A strain was very likely to be detected in a meat sample obtained from the Coles shop in Belconnen, but unlikely to be observed in a meat sample from the Coles store in Gungahlin. Chicken mince is made up of the meat from many birds that for a variety of reasons are unsuitable for other uses. Overall, phylogroup B1 strains are the second most common group of strains observed across all meat samples, yet B1 strains are less likely to be detected in mince than in other meat types (Fig. 2.3). Conversely, B2 strains are the least common group of strains, but are far more likely to be detected in mince than in other meat types (Fig. 2.4). B2 strains are the most common strains to be isolated from humans living in the Canberra region (Gordon et al., 2005, Blyton et al., 2014, Gordon et al., 2015) and perhaps the high frequency of B2 strains in mince is a consequence of contamination from processing plant workers. The probability of detecting a phylogroup E strain varied with season regardless of meat type, retailer or locality. By

contrast, the probability of detecting phylogroup F strains varied in a complicated manner with store, season and meat type.

The frequency with which strains were resistant to either cefazolin or amoxicillin/clavulanic acid varied with the meat type from which they were recovered, with mince having high frequency of resistant strains, breast meat with low frequency of resistant strains, and thigh, pelvic region and wing meat an intermediate frequency of resistant strains. This pattern cannot be explained by the phylogroup membership of the strain most likely to be detected in a meat type (e.g. B2 in mince) or the number of strains typically detected in a meat type. Nalidixic acid resistance also varied with meat type, with strains isolated from mince and pelvic region meat more likely to be resistant than strains from other meat types. Nalidixic acid was significantly more likely to be observed among strains collected in summer compared to those isolated from the meat samples collected in winter. Rearing method also influenced the probability of a strain being resistant to one or more antibiotics. Although why such high frequencies of antibiotic resistant *E. coli* strains are detected in meat samples from organically raised birds is unknown, as the meat products cannot be sold as organic if antibiotics were used in barns and farms during rearing of these birds.

In Australia, fluoroquinolones cannot be used in food-producing animals (Cheng et al., 2012). This study found that 14 (4.6%) of 306 meat samples harboured a fluoroquinolone resistant strain of *E. coli*. Fluoroquinolone use cannot explain the presence of these resistant strains in poultry meat. Nonetheless, if these figures are extrapolated country wide, it would suggest that poultry are a significant reservoir of fluoroquinolone resistance determinants.

The results of this study demonstrate that poultry meat products are likely to be contaminated with a genetically diverse community of *E. coli* and that the presence of *E. coli* in a sample is not only a consequence of contamination of the meat by the bird's own faecal *E. coli*, but that contamination may occur at multiple points in the production and distribution chain.

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2.7 Supplemental Materials

Statistical Analyses: Approach and Results

Presence of *E. coli* and number of REP-types in a sample

In the first analysis we investigated which factors influenced the presence/absence and richness in the conventional meat samples. Samples taken from the breast meat of whole carcasses were excluded from these analyses as they were only sampled in the winter. The explanatory variables were (1) meat type; (2) retailer; (3) town-centre; (4) season; and (5) store (which was a two-way interaction between retailer and town-centre). Selected two way interactions between the explanatory variables were also considered in the case of the richness analysis. Non-significant explanatory variables were excluded by backwards elimination.

Supplemental Table 2.1. All explanatory variables of the probability that an *E. coli* strain was detected in a conventional meat sample

Explanatory variable	Order eliminated	P value[1]
Meat type	N/A	**0.021**
Retailer	N/A	**<0.001**
Town-centre	2	0.303
Season	3	0.060
Store (Retailer x Town-centre)	1	0.263

1. Type 3 probability in final model in which the explanatory variable was included

Supplemental Table 2.2. All explanatory variables of richness in conventional meat samples

Explanatory variable	Order eliminated	P value[1]
Meat type	N/A	**<0.001**
Retailer	N/A	**<0.001**
Town-centre	7	0.5969

Season	N/A	0.3941
Store (Retailer x Town-centre)	6	0.2067
Meat type x Retailer	N/A	**<0.001**
Meat type x Town-centre	2	0.5392
Meat type x Season	N/A	**0.0380**
Meat type x Store	1	0.2816
Season x Retailer	5	0.3639
Season x Town-centre	4	0.6259
Season x Store	3	0.1124

1. Type 3 probability in final model in which the explanatory variable was included

Supplemental Table 2.3. All explanatory variables of richness in organic breast meat samples

Explanatory Variable	**Order eliminated**	**P value when eliminated**
Retailer	**N/A**	**0.011**
Town-centre	5	0.272
Season	4	0.636
Store (Retailer x Town-centre)	2	0.211
Season x Retailer	1	0.089
Season x Town-centre	3	0.460

1. Type 3 probability in final model in which the explanatory variable was included

Supplemental Table 2.4. All explanatory variables of richness in free-range breast meat samples

Explanatory Variable	**Order eliminated**	**P value when eliminated**
Retailer	3	0.879
Town-centre	**N/A**	**0.026**
Season	5	0.605

Store (Retailer x Town-centre)	2	0.259
Season x Retailer	1	0.316
Season x Town-centre	4	0.184

1. Type 3 probability in final model in which the explanatory variable was included

Distribution of phylogenetic groups (phylogroups)

To determine which factors influenced the phylogroups detected in the meat samples that contained *E. coli*, we fitted generalised linear regression models (family = binomial) using the statistical package in R (http://www.R-project.org/). It was assumed that the different strains of *E. coli* in a sample did not compete, as it was unlikely that the strains were growing in the samples given they are stored at 5^{o}C. Further, *E. coli* cell densities in these meat samples was also low, as *E .coli* was seldom detected in a homogenate of a meat sample prior to the sample's enrichment. Finally, as an average of 19 isolates were obtained from each sample, there was the potential for all *E. coli* phylogroups to be detected in a sample. For these reasons, the detection of one phylogroup in a sample was considered independent of the detection of the other phylogroups, and thus, the different phylogroups were analysed in separate analyses. The response variables in the analyses were whether or not the phylogroup in question was detected in a sample that had *E. coli*. The REP-type richness of the samples was included as a covariate as it was expected that the probability of detecting a phylogroup would increase as the number of strains detected increased.

In the first analysis we investigated which factors influenced the phylogroups detected in the conventional meat samples. Samples taken from the independent butcheries were excluded from this analysis as there were too few genotypes recovered from these meat samples for meaningful statistical comparisons. Samples taken from the breast meat of whole carcasses were also excluded from this analysis. The explanatory variables were (1) meat type; (2) retailer; (3) town-centre; (4) season; and (5) store. Selected two way interactions between the explanatory variables were also considered. Non-significant explanatory variables were excluded by backwards elimination.

In the third analysis, we determined whether the rearing method influenced whether each of the phylogroups were detected in a breast meat sample. Only samples from Coles and Woolworths were included in this analysis. The explanatory variable was the rearing method i.e. conventional, organic or free-range.

In the fourth analysis, we investigated which factors influenced whether each of the phylogroups were detected in the organic and free-range breast meat samples. The explanatory variables were (1) retailer; (2) town-centre; (3) season; and (4) store. Selected two way interactions between the explanatory variables were also considered. Non-significant explanatory variables were excluded by backwards elimination.

Supplemental Table 2.5. All explanatory variables of the probability of detecting a phylogroup B1 strain in a conventional meat sample from the major retailers.

Explanatory Variable	Order eliminated	P value when eliminated
REP type richness	**N/A**	**0.001**
Meat type	**N/A**	**0.016**
Retailer	6	0.285
Town-centre	9	0.322
Season	8	0.443
Store (Retailer x Town-centre)	2	0.149
Meat type x Retailer	3	0.066
Meat type x Town-centre	4	0.083
Meat type x Season	5	0.185
Season x Retailer	1	0.161
Season x Town-centre	7	0.257

Supplemental Table 2.6. All explanatory variables of the probability of detecting a phylogroup B2 strain in a conventional meat sample from the major retailers

Explanatory Variable	Order eliminated	P value when eliminated
REP type richness	**N/A**	**0.001**
Meat type	**N/A**	**<0.001**
Retailer	5	0.418
Town-centre	4	0.635
Season	7	0.869
Store (Retailer x Town-centre)	3	0.103
Meat type x Season	6	0.115
Season x Retailer	2	0.254
Season x Town-centre	1	0.405

Supplemental Table 2.7. All explanatory variables of the probability of detecting a phylogroup A strain in a conventional meat sample from the major retailers

Explanatory Variable	Order eliminated	P value when eliminated
REP type richness	**N/A**	**<0.001**
Meat type	7	0.189
Retailer	N/A	0.178
Town-centre	N/A	0.084
Season	5	0.229
Store (Retailer x Town-centre)	**N/A**	**0.022**
Meat type x Retailer	6	0.198
Meat type x Town-centre	4	0.210
Meat type x Season	1	0.475

Season x Retailer	3	0.127
Season x Town-centre	2	0.396

Supplemental Table 2.8. All explanatory variables of the probability of detecting a phylogroup D strain in a conventional meat sample from the major retailers

Explanatory Variable	Order eliminated	P value when eliminated
REP type richness	**N/A**	**<0.001**
Meat type	4	0.924
Retailer	8	0.575
Town-centre	7	0.349
Season	6	0.732
Store (Retailer x Town-centre)	2	0.685
Meat type x Retailer	3	0.570
Season x Retailer	1	0.928
Season x Town-centre	5	0.239

Supplemental Table 2.9. All explanatory variables of the probability of detecting a phylogroup E strain in a conventional meat sample from the major retailers

Explanatory Variable	Order eliminated	P value when eliminated
REP type richness	**N/A**	**0.008**
Meat type	8	0.467
Retailer	9	0.292
Town-centre	7	0.880
Season	**N/A**	**0.033**

Store (Retailer x Town-centre)	6	0.437
Meat type x Retailer	2	0.552
Meat type x Town-centre	1	0.712
Meat type x Season	8	0.340
Season x Retailer	3	0.596
Season x Town-centre	5	0.533

Supplemental Table 2.10. All explanatory variables of the probability of detecting a phylogroup F strain in a conventional meat sample from the major retailers

Explanatory Variable	**Order eliminated**	**P value when eliminated**
REP type richness	**N/A**	**<0.001**
Meat type	N/A	0.199
Retailer	N/A	0.739
Town-centre	N/A	0.423
Season	N/A	0.307
Store (Retailer x Town-centre)	2	0.139
Meat type x Retailer	3	0.136
Meat type x Town-centre	1	0.371
Meat type x Season	**N/A**	**0.042**
Season x Retailer	**N/A**	**0.022**
Season x Town-centre	**N/A**	**0.009**

Supplemental Table 2.11. All explanatory variables of the probability of detecting a phylogroup A strain in a free-range or organic meat sample

Explanatory Variable	Order eliminated	P value when eliminated
REP type richness	**N/A**	**0.029**
Retailer	5	0.925
Town-centre	3	0.426
Season	6	0.187
Store (Retailer x Town-centre)	1	0.941
Season x Retailer	2	0.908
Season x Town-centre	4	0.173

Supplemental Table 2.12. All explanatory variables of the probability of detecting a phylogroup B1 strain in a free-range or organic meat sample

Explanatory Variable	Order eliminated	P value when eliminated
REP type richness	**N/A**	**<0.001**
Retailer	**N/A**	**0.051**
Town-centre	2	0.312
Season	1	0.368

Supplemental Table 2.13. All explanatory variables of the probability of detecting a phylogroup B2 strain in a free-range or organic meat sample

Explanatory Variable	Order eliminated	P value when eliminated
REP type richness	**4**	**0.568**
Retailer	2	0.632
Town-centre	1	0.942
Season	3	0.670

Supplemental Table 2.14. All explanatory variables of the probability of detecting a phylogroup D strain in a free-range or organic meat sample

Explanatory Variable	Order eliminated	P value when eliminated
REP type richness	**N/A**	**0.599**
Retailer	**N/A**	**0.017**
Town-centre	1	0.679
Season	**N/A**	**0.002**

Supplemental Table 2.15. All explanatory variables of the probability of detecting a phylogroup E strain in a free-range or organic meat sample

Explanatory Variable	Order eliminated	P value when eliminated
REP type richness	**N/A**	**0.181**
Retailer	1	0.972
Season	2	0.314

Supplemental Table 2.16. All explanatory variables of the probability of detecting a phylogroup F strain in a free-range or organic meat sample

Explanatory Variable	Order eliminated	P value when eliminated
REP type richness	**N/A**	**0.600**
Retailer	2	0.125
Season	1	0.315

Antimicrobial resistance

Isolates that were classified as intermediate according to their inhibition zone diameters were generally grouped with the resistant isolates for analysis. However, isolates classified as intermediate to nitrofurantoin, ampicillin, or cefazolin were grouped with the susceptible isolates following Blyton et al. (2015).

Resistance is often correlated among different antibiotics that either belong to the same class or because the genes that encode resistance are co-located on the same integon gene cassettes. Therefore, we performed a multiple correspondence analysis in TANAGRA to identify and summarise any associations between the insistences of resistance to different antibiotics. Only antibiotics for which resistance was observed in greater than 5% of REP-types isolates were included in the analysis. Significant contributors to each axis were identified using v-tests with the standard critical cut off of 4.

To investigate which factors influenced the incidence of antibiotic resistance among the *E. coli* isolates we fitted a series of generalised linear regression models (family = binomial) using the stats package in R (http://www.R-project.org/). The response variables in the analyses were whether or not a particular unique REP type (within a sample) was resistant to one or more of a group of antibiotics. Antibiotics that clustered together in the multiple correspondence analysis were grouped together. The

phylogroups of the isolates were included as a covariate as antibiotic resistance is known to vary between the phylogroups (Skurnik et al., 2005, Johnson et al., 2009, Blyton et al., 2015).

In the first analysis we investigated which factors influenced the incidence of antibiotic resistance in *E. coli* from the conventional meat samples. Samples taken from the breast meat of whole carcasses were excluded from this analysis. The explanatory variables were (1) meat type; (2) retailer; (3) town-centre; (4) season; and (5) store. Selected two way interactions between the explanatory variables were also considered where sample sizes permitted. Non-significant explanatory variables were excluded by backwards elimination.

In the second analysis, we determined whether the rearing method influenced the incidence of antibiotic resistance in a breast meat sample. Only samples from Coles and Woolworths were included in this analysis. The explanatory variable was the rearing method i.e. conventional, organic or free-range.

Supplemental Table 2.17. All explanatory variables of the probability that an *E. coli* strain isolated from conventional meat was resistant to either amoxicillin/clavulanic acid and/or cefazolin

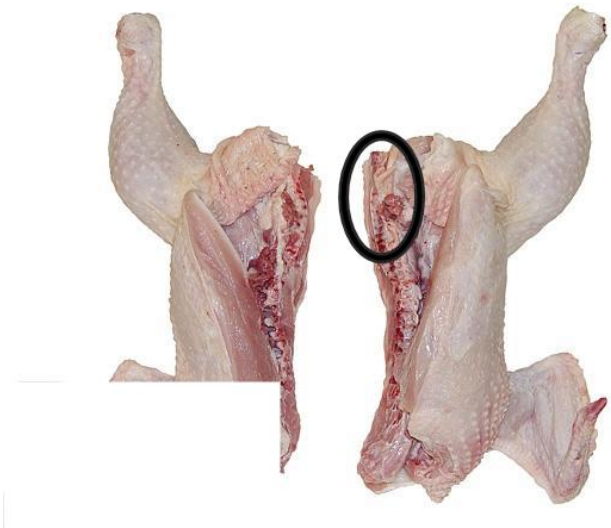
Explanatory variable	Order eliminated	P value[1]
Phylogroup	N/A	0.053
Meat type	N/A	**<0.019**
Retailer	2	0.284
Town-centre	4	0.122
Season	3	0.139
Season x Retailer	1	0.872

1. Type 3 probability in final model in which the explanatory variable was included except for phylogroup for which the Type 1 probability is given

Supplemental Table 2.18. All explanatory variables of the probability that an *E. coli* strain isolated from conventional meat was resistant to one or more of ampicillin, trimethoprim/sulfamethoxazole and tetracycline

Explanatory variable	Order eliminated	P value[1]
Phylogroup	N/A	0.054
Meat type	7	0.385
Retailer	N/A	0.821
Town-centre	N/A	0.668
Season	5	0.195
Store (Retailer x Town-centre)	N/A	**0.001**
Meat type x Retailer	6	0.078
Meat type x Town-centre	3	0.114
Meat type x Season	2	0.114
Season x Retailer	1	0.710
Season x Town	4	0.195

1. Type 3 probability in final model in which the explanatory variable was included except for phylogroup for which the Type 1 probability is given



Supplemental Figure 2.1. Pelvic region (marked in black oval) of whole chicken.

Chapter 3

Genetic structure of *Escherichia coli* in poultry meat from Canberra, Australia

3.1 Abstract

Escherichia coli is a highly heterogeneous Gram negative Enterobacteriaceae, with vast genetic variability. Except for humans, few studies have looked in depth on the variation of *E. coli* in any other species. Though some lineages like STs (sequence types) 95, 131, 69 of major phylogroups B2 and D have been well characterized, a number of other lineages like STs 88, 57, 117 of minor phylogroups (C, E and F respectively) are still unclear. In this study, whole-genome based approach was used for understanding the genetic structure of *E. coli* in poultry meat. In silico typing methods were used for ST, serotype, resistance, and virulence factors (VF) determination. Identical phylogroups with similar STs were often detected across different meat types, shops, town centres, and even seasons. Phylogroup A was over-represented in poultry meat with 92 strains, followed by B1 and D. Phylogroups B2, F, E, cryptic clade I and C were detected less frequently, as in the order given. Out of 283 strains, 64% harboured at least one plasmid-mediated resistance determinants out of which 61.3% were multi-drug resistant, and 80.9% had plasmid incompatibility groups. Phylogroup B2 strains had the highest virulence determinants (with 48.6% ExPEC status) with low resistance, while STs like F-ST354, A-ST3333 and A-ST6053 were found to be highly multi-drug resistant. Resistance to critically important antimicrobials of 3rd generation cephalosporins and fluoroquinolones was low compared to other countries (Johnson et al., 2007, Kluytmans et al., 2013), and non-existent for carbapenems. No two phylogroups shared common STs, while serotype overlap was seen in strains from different phylogroups.

Overall, there was relatively little ST diversity in that many STs were seen multiple times, while serotype diversity within STs was high when compared to human isolates. Clonal groups with potential ExPEC traits like A-CC93, B1-CC155, C-CC23, D-CC349, E-CC350 were also identified. Most of the VF diversity appears to be driven at the ST level rather than phylogroup, where some of the STs had very different VF profiles. Furthermore, APEC genes were present in 28.3% of strains and overrepresented in specific lineages belonging to A-CC665, B2-ST95 and ST131, E-CC350 and also, F-ST117. Phylogroup E strains were more likely to be APEC-associated compared to other phylogroups like B1 and D.

3.2 Introduction

Escherichia coli (E. coli) is a rod-shaped, facultative anaerobic, Gram negative bacterium of the family *Enterobacteriaceae*. The organism is part of the normal gut flora of warm-blooded animals and commonly found present in the lower intestine (Kaper et al., 2004). *E. coli* cells are also commonly isolated from water, sediments, food, and soil which successfully serve as a secondary habitat for this organism (Leimbach et al., 2013). Faecal-oral transmission is the major route of entry for pathogenic strains of *E. coli* to humans (Tauxe, 1998). *E. coli* is highly heterogeneous and can range from commensal groups, part of the normal flora, to highly virulent or pathogenic strains which are known to cause both intestinal and extra-intestinal infections (Kaper et al., 2004). Intestinal pathogenic *E. coli* (IPEC) variants include enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), and enteroaggregative *E. coli* (EAEC) (Kaper et al., 2004).

Additionally, extra-intestinal pathogenic *E. coli* (ExPEC) strains can successfully colonize the GI tract of healthy human hosts (Johnson et al., 2008). When *E. coli* leaves its natural habitat (gastrointestinal or GI tract) and enters other organs or sites (like urinary tract, bloodstream), cause infections like urinary tract infection (UTI) and more severe infections of the bloodstream (septicemia or bacteremia), neonatal meningitis, pneumonia (especially in immunocompromised patients), sepsis, and many other infections (Johnson and Russo, 2002). In fact, *E. coli* is known to cause more than 90% of UTIs worldwide, a majority of which are seen in women (Kucheria et al., 2005). In addition, avian pathogenic *E. coli* (APEC) are known to cause systemic infections like colibacillosis, a big problem of the poultry industry (Dho-Moulin and Fairbrother, 1999). APEC closely resembles certain human ExPEC variants, which brought about the question if human ExPEC infections have food-animal reservoirs or even origin (Manges, 2016).

Poultry meat as a foodborne reservoir of zoonotic *E. coli,* which is often multi-drug resistant (MDR), has been widely studied (Vincent et al., 2010, Johnson et al., 2005, Kluytmans et al., 2013, Jakobsen et al., 2010, Overdevest et al., 2011, Manges, 2016).

The meat has been indicated to harbour strains most closely resembling human ExPEC strains compared to other meats like beef and pork. They often exhibit MDR to important antimicrobials belonging to classes like fluoroquinolones (FQ), cephalosporins and beta-lactamases, collectively termed as extended-spectrum beta-lactamases or ESBL (Manges and Johnson, 2015, Johnson et al., 2009, Bergeron et al., 2012). With the ability of *E. coli* to transfer both resistance and virulence genes, not only through vertical transfer but also commonly through horizontal transfer, the threat concern and relevance is much higher than initially given (Manges, 2016). The association of *E. coli* with foodborne diarrhea has been widely characterized and focused on, while the ability of the organism to cause extra-intestinal infections was not as clearly defined before early the 2000's (Johnson and Russo, 2002). Pathogenic *E. coli* is often associated with virulence factors (VFs) which aid in adhesion (*afa/draB, papAH, papC, papG, fimC, fimH, focG, hra, iha, tsh, sfa/foc*), invasion (*tia*, *kpsE*, *gimB*, *ibeA*), toxins and hemolysins (*astA, sat, stx_1, stx_2, cdtB, hlyA*), iron-acquisition systems (*chuA, fyuA, iroN, ireA, irp2, iucC, iutA, sitD*), colicins (*cva, cba, cma*), polysaccharide subunits (*kpsMT*), serum resistance and survival (*neuC, ompA, ompT, traT, iss*) (Johnson et al., 2003, Smith et al., 2007).

With the advent of whole genome sequencing, the decrease in costs, and a reference as the new "gold standard" in phylogenetic studies, a number of research groups are shifting towards this approach (Leimbach et al., 2013). In STs, serotypes, resistance and virulence determinants can now be easily carried out even with basic Bioinformatics skills using publicly available websites like Centre for Genomic Epidemiology (CGE) http://www.genomicepidemiology.org/ hosted in Denmark (Larsen et al., 2012), programs like MAUVE (Darling et al., 2010) and many more. This can help eliminate or reduce the use of a number of molecular typing techniques.

In this study, 283 poultry meat strains were selected for whole genome sequencing and in silico characterization was performed to group them using in silico typing to their respective STs, serotypes/ serogroups and also determined their virulence and resistance determinants.

3.3 Materials and Methods

3.3.1 *Strains*

Poultry meat (n=306) were sampled from sixteen shops representing four major town centres and independent butchers in Canberra, Australian Capital Territory (ACT), Australia on November 2013, April 2014 and August 2014. These dates represented sampling from summer, autumn and winter seasons respectively. Breast fillet, thigh fillet, mince, wings and whole meat were sampled and processed within an hour from sampling time. *E. coli* was isolated using enrichment and antibiotic selection, characterized then further molecular characterization was carried out on 3415 isolates. The isolates were assigned to phylogroups (phylogenetic groups) A, B1, B2, C, D, E, F or clade I as per Clermont et al., 2013 then REP (Repetitive element palindromic)-PCR typing was performed to classify as strains using ERIC (Versalovic et al., 1991) and CGG primers (Adamus-Bialeket al., 2009). A sub-set of the strains representing the various phylogroups as determined by Clermont quadruplex method (Clermont et al., 2013) were then selected for whole genome sequencing. In choosing the strains, only strains from different meat samples were selected for WGS.

3.3.2 *Whole genome sequencing*

Whole genome sequencing was performed on the selected poultry meat strains (n=283) using Illumina MiSeq sequencing platform with 600-cycle Nextera XT version 3-reagent kit (2x300 paired-end reads). The protocols used for library preparations including DNA extraction were as per (Vangchhia et al., 2016). The raw sequences were assembled and exported as fasta files using CLC Genomics Workbench V9.0. Raw reads are available in Sequence Read Archive (SRA) hosted by National Center for Biotechnology Information (NCBI) (SRS1690743-SRS1690752).

3.3.3 *In silico typing*

The strains were typed to their respective STs using MLST University of Warwick (Achtman) scheme (Wirth et al., 2006), available on the Centre for Genomic Epidemiology (CGE) website (www.genomicepidemiology.org) (Larsen et al., 2012). The

strain with un-typeable STs on Achtman scheme were identified by uploading raw sequence output files on Enterobase (also following Achtman scheme), a majority of which were novel STs. The strains were also serotyped using SeroTypeFinder (Joensen et al., 2015) tool available on the Centre for Genomic Epidemiology (CGE) website (www.genomicepidemiology.org). The antimicrobial resistance and virulence genes were also determined using ResFinder (Zankari et al., 2012) and VirulenceFinder (Joensen et al., 2014); tools both available on the same CGE website. All strains were additionally verified for their quinolone/ fluoroquinolone resistance by uploading the fasta sequences to the website https://card.mcmaster.ca/ (McArthur et al., 2013), which provides the option of identifying chromosomal mutations conferring resistance. Multi-drug resistance (MDR) was defined as strains possessing two or more different classes of antimicrobial resistance determinants.

3.3.4 *Phylogenetic tree*

A phylogenetic tree of all the poultry meat strains was inferred using core genome single-nucleotide polymorphisms (SNPs) by HARVEST suite (Treangen et al., 2014). Recombination detection option in Harvest was used. The strains are further referred to their phylogenetic membership as designated using this tree in this study.

3.4 Results

3.4.1 *MLST*

Ninety-two poultry meat strains clustered under phylogroup A, 48 phylogroup B1, 37 phylogroup B2, 4 phylogroup C, 5 Clade I, 43 phylogroup D, 28 phylogroup E and 26 phylogroup F, in the phylogenetic tree extracted using core genome SNPs obtained by Harvest suite of tools (Fig. 3.1).

Out of 283 strains belonging to eight phylogroups, sixty-nine Achtman scheme STs were detected. In phylogroup A, 23 different Achtman STs were present. The distribution of Achtman STs and their clonal complexes (CCs) were ST665 (n=17, CC665), ST10 (n=16,

CC10), ST6047 (n=13, CC665), ST48 (n=8, CC48), ST6053 (n=8), ST93 [n=4, CC168/ CC93), ST206 (n=4), ST399 (n=4), ST2739 (n=2, CC48), ST3333 (n=2, CC93) and 1 each of STs 1137 (CC10), 2207, 2470 (CC10), 6050 (CC48), 6051 (CC48), 373 (CC93), 6061 (CC93), 216, 746, 1408, 2705, 3770 (CC93), 5295.

All phylogroup B1 strains were typeable with Achtman scheme (19 types). The strains and their respective STs were STs 58 [n=7, CC155), 101 (n=4, CC101), 155 (n=8, CC155), 212(n=5), 295 (n=3), 345 (n=2), 453 (n=2, CC86), 602 (n=2, CC446), 641 (n=2, CC86), 683 (n=3, CC155), 6062 (n=2, CC155), and 1 each of STs 224 (CC224), 297, 906 (CC224), 949 (CC155), 1125, 1841, 3190 and 6046. In phylogroup B2, 5 STs were identified STs 95 (n=17), 131 (n=6), 135 (n=11), 355 (n=2) and 372 (n=1). Four of the phylogroup C strains all belonged to ST88 and the 5 clade I strains belonged to ST770. Out of the 43 strains belonging to phylogroup D, 13 STs were present and one was un-typeable but clustered closest with ST1158 using Achtman scheme. The STs according to their prevalence were STs 69 (n=9), 38 (n=7, CC38), 68 (n=5), 115 (n=4, CC115), 349 (n=4, CC349), 2309 (n=3, CC115), 315 (n=2, CC38), 1158 (n=2), 1166 (n=2), and 1 each of STs 362, 405, 714, and 6066 (CC349).

Majority of phylogroup E strains (85.7%) belonged to CC350 and were of ST57 (n=21, CC350) and ST371 (n=3, CC350). Additionally, ST219 (n=2), and 1 strain each of STs 1011 and 5281 were also present. Five Achtman STs were present in total. Two Achtman STs represented phylogroup F, ST354 (n=9) and ST117 (n=17). ST354 strains were all resistant to fluoroquinolone (ciprofloxacin and nalidixic acid) and two ST117 strains exhibited nalidixic acid resistance (Vangchhia et al., 2016, Chapter 4).

In addition, 289 human isolates also collected from Canberra region were analysed for their phylogroup and ST distribution. Among the major phylogroups, 94 isolates belonged to phylogroup A with 44 STs, 49 to phylogroup B1 with 34 STs, 168 to phylogroup B2 belonging to 40 STs, and 32 to phylogroup D with 14 STs. Meanwhile among the minor phylogroups, 7 isolates were of phylogroup C belonging to 3 diferrent STs, 2 were of phylogroup E with both belonging to different STs, and then 29 belonged

to phylogroup F with 8 different STs (Supplemental Table 3.2). The strains will further be referred to as per their Achtman scheme MLST in this paper.

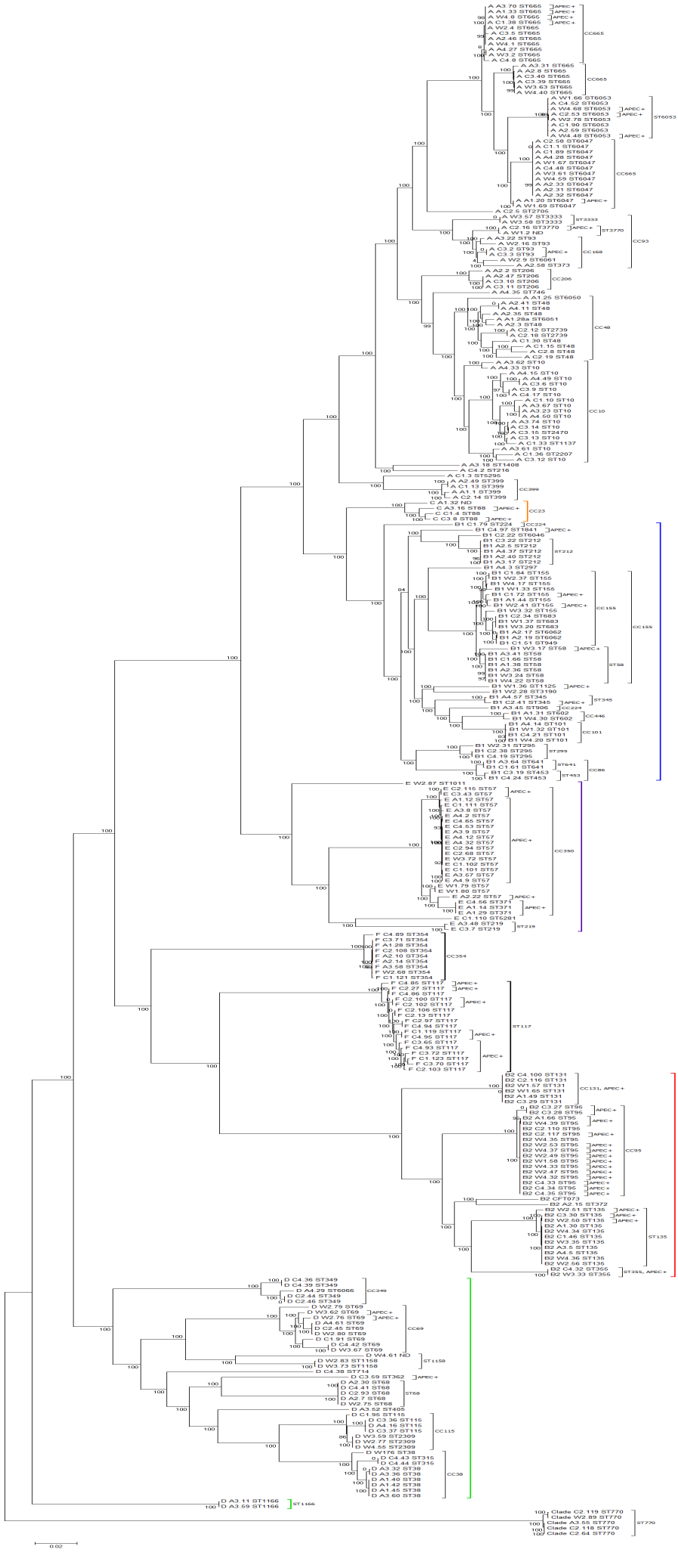

A A3 70 ST665
A A1 33 ST665
A W4.8 ST665
A C1 38 ST665
A W2.4 ST665
A C3.5 ST665
A A2.46 ST665
A W4.1 ST665
A A4.27 ST666
A W3.2 ST666
A C4.8 ST665
A A3.31 ST665
A A2.8 ST665
A C3.40 ST665
A C3.39 ST665
A W3.63 ST665
A W4.40 ST665
CC665
A W1.66 ST6053
A C4.52 ST6053
A W4.68 ST6053
A C2.53 ST6053
A W2.78 ST6053
A C1.90 ST6053
A A2.59 ST6053
A W4.48 ST6053
ST6053
A C2.58 ST6047
A C1.1 ST6047
A C1.89 ST6047
A A4.28 ST6047
A W1.67 ST6047
A C4.48 ST6047
A W3.61 ST6047
A W4.59 ST6047
A A2.33 ST6047
A A2.31 ST6047
A A2.32 ST6047
A A1.20 ST6047
A W1.69 ST6047
A C2.5 ST2706
A W3.57 ST3333
A W3.58 ST3333
ST3333
A C2.16 ST3770
A W1.2 ND
ST3770
A A3.22 ST93
A W2.16 ST93
A C3.2 ST93
A C3.3 ST93
A W2.9 ST6061
A A2.58 ST373
CC93
CC168
A A2.2 ST206
A A2.47 ST206
A C3.10 ST206
A C3.11 ST206
A A4.35 ST746
CC206
A A1.25 ST6050
A A2.41 ST48
A A4.11 ST48
A A2.35 ST48
A A1.28a ST6051
A A2.3 ST48
A C2.12 ST2739
A C2.18 ST2739
A C1.30 ST48
A C1.15 ST48
A C2.8 ST48
A C2.19 ST48
CC48
A A3.62 ST10
A A4.33 ST10
A A4.15 ST10
A A4.49 ST10
A C3.6 ST10
A C3.9 ST10
A C4.17 ST10
A C1.10 ST10
A A3.67 ST10
A A3.23 ST10
A A4.50 ST10
A A3.74 ST10
A C3.14 ST10
A C3.15 ST2470
A C3.13 ST10
A C1.33 ST1137
A A3.61 ST10
A C1.36 ST2207
A C3.12 ST10
CC10
A A3.18 ST1408
A C4.2 ST216
A C1.3 ST6295
A A2.49 ST399
A C1.13 ST399
A A1.1 ST399
A C2.14 ST399
CC399
C A1.32 ND
C A3.16 ST88
C C1.4 ST88
C C3.8 ST88
CC23
B1 C1.79 ST224
CC224
B1 C4.97 ST1841
B1 C2.22 ST6046
B1 C3.22 ST212
B1 A2.5 ST212
B1 A4.37 ST212
B1 A2.40 ST212
B1 A3.17 ST212
B1 A4.3 ST297
ST212
B1 C1.84 ST155
B1 W2.37 ST155
B1 W4.17 ST155
B1 W1.33 ST155
B1 C1.72 ST155
B1 A1.44 ST155
B1 W2.41 ST155
B1 W3.32 ST155
B1 C2.34 ST683
B1 W1.37 ST683
B1 W3.20 ST683
B1 A2.17 ST6062
B1 A2.19 ST6062
B1 C1.51 ST949
B1 W3.17 ST58
CC155
B1 A3.41 ST58
B1 C1.46 ST58
B1 A1.38 ST58
B1 A2.36 ST58
B1 W3.24 ST58
B1 W4.22 ST58
ST58
B1 W1.36 ST1125
B1 W2.28 ST3190
B1 A4.57 ST345
B1 C2.41 ST345
B1 A2.45 ST906
ST345
B1 A1.31 ST602
B1 W4.30 ST602
CC446
B1 A4.14 ST101
B1 W1.32 ST101
B1 C4.21 ST101
B1 W4.20 ST101
CC101
B1 W2.31 ST295
B1 C2.38 ST295
B1 C4.19 ST295
ST295
B1 A3.64 ST641
B1 C1.61 ST641
ST641
B1 C3.19 ST453
B1 C4.24 ST453
ST453
CC86
E W2.87 ST1011
E C2.115 ST57
E C3.43 ST57
E A1.12 ST57
E C1.111 ST57
E A3.8 ST57
E A4.2 ST57
E C4.65 ST57
E C4.63 ST57
E A3.9 ST57
E A4.12 ST57
E A4.32 ST57
E C2.94 ST57
E C2.68 ST57
E W3.72 ST57
E C1.102 ST57
E C1.101 ST57
E A3.57 ST57
E A4.9 ST57
E W1.79 ST57
E W1.80 ST57
E A2.22 ST57
E C4.56 ST371
E A1.14 ST371
E A1.29 ST371
E C1.110 ST5281
APEC+
CC330
E A3.48 ST219
E C3.7 ST219
ST219
F C4.89 ST354
F C3.71 ST354
F A1.28 ST354
F C2.109 ST354
F A2.10 ST354
F A2.14 ST354
F A3.58 ST354
F W2.68 ST354
F C1.121 ST354
CC354
F C4.85 ST117
F C2.27 ST117
F C4.86 ST117
F C2.100 ST117
F C2.102 ST117
F C2.106 ST117
F C2.13 ST117
F C2.97 ST117
F C4.94 ST117
F C1.119 ST117
F C4.96 ST117
F C3.65 ST117
F C4.93 ST117
F C3.72 ST117
F C1.123 ST117
F C3.70 ST117
F C2.103 ST117
ST117
B2 C4.100 ST131
B2 C2.116 ST131
B2 W1.57 ST131
B2 W1.65 ST131
B2 A1.49 ST131
B2 C3.29 ST131
CC131, APEC+
B2 C3.27 ST95
B2 C3.28 ST95
B2 A1.66 ST95
B2 W4.39 ST95
B2 C2.110 ST95
B2 C2.117 ST95
B2 W4.35 ST95
B2 W2.53 ST95
B2 W4.37 ST95
B2 W2.49 ST95
B2 W1.58 ST95
B2 W4.33 ST95
B2 W2.47 ST95
B2 W4.32 ST95
B2 C4.33 ST95
B2 C4.34 ST95
B2 C4.36 ST95
CC95
B2 CF1073
B2 A2.16 ST372
B2 W2.51 ST135
B2 C3.30 ST135
B2 W2.50 ST136
B2 A1.30 ST135
B2 W4.34 ST135
B2 C1.46 ST135
B2 W3.35 ST135
B2 A3.5 ST135
B2 A4.5 ST135
B2 W4.36 ST136
B2 W2.66 ST136
B2 C4.32 ST365
B2 W3.33 ST355
ST135
ST355, APEC+
D C4.36 ST349
D C4.39 ST349
D A4.29 ST6066
D C2.44 ST349
D C2.46 ST349
CC349
D W2.79 ST69
D W3.62 ST69
D W2.76 ST69
D A4.61 ST69
D C2.45 ST69
D W2.80 ST69
D C1.91 ST69
D C4.42 ST69
D W3.67 ST69
D W4.61 ND
CC69
D W2.83 ST1158
D W3.73 ST1158
ST1158
D C4.38 ST714
D C3.59 ST362
D A2.30 ST68
D C4.41 ST68
D C2.93 ST68
D A2.7 ST68
D W2.75 ST68
ST68
D A3.52 ST405
D C1.95 ST115
D C3.36 ST115
D A4.16 ST115
D C3.37 ST115
D W3.59 ST2309
D W2.77 ST2309
D W4.55 ST2309
CC115
D W176 ST38
D C4.43 ST316
D C4.44 ST316
D A3.32 ST38
D A3.36 ST38
D A1.40 ST38
D A1.42 ST38
D A1.45 ST38
D A3.60 ST38
CC38
D A3.11 ST1166
D A3.59 ST1166
ST1166
Clade C2.119 ST770
Clade W2.89 ST770
Clade A3.55 ST770
Clade C2.118 ST770
Clade C2.64 ST770
ST770
0.02

Figure 3.1: Phylogenetic tree representing all 283 strains inferred using core genome SNPs by HARVEST. Clearly defined branching is observed based on phylogroup distribution. The respective phylogroups with their respective STs and CCs (when applicable) are as labelled and phylogroups are indicated as A = yellow bracket, B1 = blue bracket, B2 = red bracket, C = orange bracket, D = green brackets, E = purple bracket, F = **bold black** brackets, and clade I = grey bracket. Different zoomed sections of the tree presented in Supplemental Material (Fig. 3.1a to 3.1e).

3.4.2 *Serotype diversity*

Out of 283 strains, 39.2% (n=111) of the strains had un-typeable O group where 61 unique serogroups i.e. O antigen types (somatic, lipopolysaccharide) were detected (Supplemental Table 3.1). It was then followed by O1 (n=13), 10 each of O15, O45, 9 each of O25, O50/O2, 8 each of O115, O2, 6 each of O11, O182, O8, 4 each of O17/O44, O18ac, O78, 3 each of O100, O132, O21, O29, O49, O6, O7, O71, O9, 2 each of O111, O13/O135, O16, O17/O77, O23, O26, O5, O53, O82, O86, and 1 each of O9, O103, O109, O117, O119, O127, O128ac, O13, O138, O139, O143, O148, O149, O150, O159, O160, O161, O166, O173, O177, O18, O180, O185, O3, O69, O83, O96 and OgC4/O118/O151 by prevalence. Thirty-four unique H (flagellar) antigen types were present namely H4 (n=62), H25 (n=22), H7 (n=18), H16 (n=17), H1 (n=12), 11 each of H34, H9, 10 each of H10, H18, H6, 9 of H21, 8 each of H15, H19, H37, H49, 7 each of H11, H12, 6 each of H32, H5, H8, 4 each of H48, H51, 3 each of H31, H52, 2 each of H2, H30, H45, and 1 each of H17, H20, H23, H26, H28, H42, and only 1 strain was un-typeable.

Serotype diversity was highly variable in poultry meat strains of different phylogroups (Supplemental Table 3.1). There were instances where a single ST (say, ST117, n = 17) belonged to 10 different serotypes. Serotype O25:H4 was shared between two phylogroups B2 (ST131 strains) and D (W4-61, an un-typeable strain that clustered with ST1158 strains). Also O45:H19 was shared with a strain from phylogroup A (ST6047) and three strains from phylogroup E of ST371. For strains with both O and H antigens typeable, no other overlaps existed of similar serotypes between different phylogroups other than these two serotypes.

3.4.3 *Virulence factors (VFs) distribution*

Overall, VF number varied with phylogorup, where phylogroup A had an average of 15.3 VFs and B1 had 14.3, detected using VirulenceFinder. Meanwhile, phylogroup B2 had an overall average VFs of 27.8 and was highest among all the phylogroups, followed by clade I with 24.6, phylogroup C with 22.2, phylogroup E with 21.7, phylogroup F with 21.1 and phylogroup D with an average of 17. Phylogroup A-CC10 had an average of 11.9 virulence factors (VFs) for 69 genes analysed. CC48 had an average of 11.7, CC93 had 19.7, ST206 had 8, ST399 had 5.2, ST665 (CC665) had 20.1, ST6047 (CC665) had 18.8, ST6053 had 20.9 and individual strains of STs 216, 746, 1408, 2705 and 5295 had 4, 10, 5, 15 and 7 respectively (Supplemental Table 3.1). A high variation in VF content was observed among strains of the same phylogorup A.

The average virulence determinants of phylogroup B1 specific lineages were lower compared to other phylogroups. CC86 had 13.7 VFs, CC101 had 14.5, CC155 had 13.6, CC224 had 16, ST212 had 14.8, ST295 had 9.7, ST345 had 17, CC446-ST602 had 16, and single strain STs like ST297 had 23, ST1125 had 19, ST1841 had 22, ST3190 had 7, and ST6046 had 14. The commonly shared virulence determinants among the major lineages were *fimH*, *eaeH, gad* and *upaG.* In phylogroup B2 strains, ST95 had an average of 30.1 VFs, ST131 had 29.5, ST135 had 23.8, ST355 had 30, and a single strain of ST372 had 16. A detailed study on ST95 and ST131, in relation to genetic comparison between human and poultry meat was presented in Chapter 4. Only ST355 and ST372 strains had *lpfA_LF82*, and *neuC* was present only in ST355 strains. In addition, ExPEC-related genes as per Johnson et al., 2003 (>/= 2 *sfa/foc*, *afa/dra*, *papA/C*, *kpsMT* II, *iutA*) were overrepresented in all ST95 strains. Furthermore, APEC-associated genes as per Johnson et al., 2008 (namely *iutA*, *hlyF*, *iss*, *iroN*, and *ompT*) were present in all ST131 and ST355 strains, 27.3% of ST135 and in 88% of ST95 strains (Excel worksheet 3.1).

CC38 of phylogroup D had an average of 10.8, CC115 had 21.4, CC349 had 18.4, ST68 had 13.4, and ST69 had 19.1. ST1158 had 23, ST1166 had 10, and single strain STs of 362, 405 and 714 had 22, 16 and 23 respectively. Detailed genetic structure study of ST69 is also presented in Chapter 4, where *eilA* and *air* (Salmonella *hilA* gene homolog) (Sheikh

et al., 2006) were identified in 88.9% and 55.5% of strains respectively (Excel worksheet 3.1).

In phylogroup E, CC350 (n=24) had 22.7 average virulence determinants, ST219 (n=2) had 20, and single strain STs 1011 and 5281 had 7 and 18 each respectively. In CC350 strains, *etsC*, *fimH*, *iroN*, *lpfA_LF82*, *omp*, *ompT*, *sitA*, *traT*, *eaeH*, *gad* and *microcin H47* were present in all strains. VFs that were over-represented were *hra* (95.8%), *iss* (95.8%), *iucC/ iutA* (91.7%), *cah* (91.7%), *ireA* (87.5%), *tsh* (87.5%), *tia* (79.2%), *papC* (58.3%), and *colicin M* (87.5%). Of note, genes commonly overrepresented in APEC isolates (Johnson et al., 2008) were detected in 75% of phylogroup E strains which all belonged to CC350 (n=21; ST57=18 and ST371=3) (Excel worksheet 3.1).

In phylogroup F, CC354 had 14.1 and ST117 had an average of 24.8, with overall average of 21.1 virulence determinants. The shared VFs between CC354 and ST117 strains were *fimH*, *iucC*, *iutA*, *sitA*, *eaeH*, *gad*, and *lpfA_B1*. Detailed genetic structure study of ST354 and ST117 were presented in Vangchhia et al., 2016 and Chapter 4 respectively. ST88 (CC23) of phylogroup C had 22.2 and clade I ST770 had 24.6 VF determinants (Excel worksheet 3.1).

Table 3.1. Virulence factors (VFs) distribution by phylogroups.

Virulence factor (VF)	Function	All strains n=283 %	A (n=92) %	B1 (n=48) %	B2 (n=37) %	C (n=4) %	D (n=43) %	E (n=28) %	F (n=26) %	Clade I (n=5) %
fimH	Adhesin	97.2	93.5	100	100	100	95.3	100	100	100
etsC	Transporter	48.8	22.2	35.4	97.3	100	30.2	92.8	65.4	100
fyuA	Iron acquisition	28.3	5.4	8.3	100	75	34.9	-	42.3	100
hra	Adhesin	54.4	67.4	29.2	35.1	25	65.1	85.7	26.9	100
ibeA	Adhesin/ Invasin	10.2	-	-	54	-	-	-	34.6	-
ireA	Iron acquisition	23.3	7.6	2.1	45.9	-	6.9	78.6	61.5	-
iroN	Iron acquisition	48	32.6	52.1	91.9	100	13.9	92.9	42.3	-
iucC	Iron acquisition	58.3	52.2	16.7	75.7	75	55.8	82.1	100	100
iutA	Iron acquisition	58.3	52.2	16.7	75.7	75	55.8	82.1	100	100
kpsE	Invasin	36.4	10.9	6.25	100	-	90.7	-	34.6	100
lpfA_LF82	Adhesin	15.9	-	29.2	8.1	-	-	100	-	-
neuC	Protectin	8.1	-	-	56.7	-	2.3	-	-	20
omp_chromo	Protectin	62.9	64.1	45.8	94.6	100	23.2	92.8	65.4	100
ompT	Protectin	63.9	48.9	62.5	97.3	100	39.5	96.4	65.4	100

papG	Adhesin	9.2	1.1	-	45.9	-	2.3	17.9	7.7	-
papC	Adhesin	19.8	2.2	8.3	48.6	50	20.9	50	7.7	100
sitA	Iron acquisition	83.4	76.1	64.6	100	100	2.3	96.4	100	100
terC	Tellurium resistance	14.5	21.7	8.3	5.4	25	27.9	-	7.7	-
traT	Protectin	72.1	56.5	81.2	97.3	100	62.8	96.4	53.8	100
usp	Toxin	16.2	-	-	100	-	-	-	34.6	-
vat	Autotransporter/ Toxin	22.9	10.9	2.1	89.2	-	4.6	7.1	65.4	-
cah_Ag43	Autotransporter	70.7	67.4	52.1	70.3	50	95.3	85.7	65.4	60
cdiA	Toxin	4.2	-	-	-	25	-	7.1	34.6	-
ybtS	Iron acquisition	28.3	5.4	8.3	100	75	34.9	-	42.3	-
ccl	Cloacin	0.7	-	-	-	-	-	-	7.7	-
iha	Adhesin	37.8	51.1	2.1	5.4	25	67.4	28.6	53.8	100
eaeH	Attaching, effacing	93.6	81.5	97.9	100	100	100	100	100	100
tia	Invasin	47	66.3	20.8	13.5	25	48.8	71.4	38.5	100
upaG	Adhesin	71.4	58.7	100	97.3	100	97.7	21.4	38.5	40
tsh	Autotransporter/ Toxin	19.4	11.9	8.3	29.7	50	4.6	75	15.4	-
arcA	Regulator	20.1	-	4.2	94.6	25	-	42.8	15.4	60
astA	Toxin	35.7	54.3	18.7	8.1	-	51.2	32.1	19.2	60
iss	Serum resistance	77	76.1	77.1	97.3	75	55.8	92.8	65.4	100
gad	Glutamate decarboxylase	93	89.1	97.9	100	100	90.7	100	84.6	80
mchF	Microcin H47 transporter	30	15.2	33.3	97.3	100	4.6	14.3	34.6	-
mchC	Microcin	2.8	-	2.1	2.7	25	2.3	-	15.4	-
mchB	Microcin H47 part of colicin H	0.7	-	-	-	-	-	-	7.7	-
lpfA_B1	Adhesin	42.4	28.3	97.9	-	100	39.5	3.6	96.1	-
celb	Colicin E2	7.4	8.7	-	-	-	20.9	-	7.7	40
tir	Type III effector	0.7	2.2	-	-	-	-	-	-	-
nleB	Effector	0.7	2.2	-	-	-	-	-	-	-
espA	Autotransporter	0.7	2.2	-	-	-	-	-	-	-
eae	Intimin	0.7	2.2	-	-	-	-	-	-	-
espB	Translocon	0.7	2.2	-	-	-	-	-	-	-
cif	Type III effector	0.7	2.2	-	-	-	-	-	-	-
hlyE	Toxin	2.5	-	-	-	-	-	-	26.9	-
hlyF	Toxin	57.2	38	58.3	94.6	100	37.2	96.4	65.4	-
pic	Autotransporter/ Toxin	3.2	-	-	-	-	-	-	34.6	-
mcmA	Microcin M part of colicin H	1.1	-	-	-	-	-	-	11.5	-
air	Enteroaggregative immunoglobulin	1.8	-	-	-	-	11.6	-	-	-
eilA	Salmonella hilA homolog	2.8	-	-	-	-	18.6	-	-	-
***ExPEC* trait**	>/= 2 *sfa/foc*, *afa/dra*, *papA/C*, *kpsMT* II, *iutA*	19.4	2.2	6.2	48.6	50	20.9	50	7.7	100
***APEC* trait**	= *iutA*, *hlyF*, *iss*, *iroN*, *ompT*	28.3	11.9	12.5	70.3	50	6.9	75	42.3	-
Col E1 (cea)	Bacteriocin	26.5	28.3	6.2	40.5	-	44.2	10.7	30.8	20
Col Ia (cia)	Toxin	35.3	18.5	41.7	83.8	50	25.6	25	26.9	100
Col Ib (cib)	Toxin	4.9	2.2	-	-	-	-	-	26.9	100
Col B (cba)	Toxin	19.4	35.9	2.1	8.1	25	27.9	3.6	7.7	40
Col M (cma)	Toxin	38.9	58.7	18.7	8.1	25	7.5	82.1	19.2	40
Microcin V (cva)	Protectin	29.3	17.4	33.3	91.9	75	4.6	17.8	26.9	-
Microcin B17	Regulator	2.5	2.2	-	-	-	9.3	-	3.8	-
Microcin H47	Col H precursor	39.9	21.7	35.4	94.6	75	6.9	89.3	38.5	-

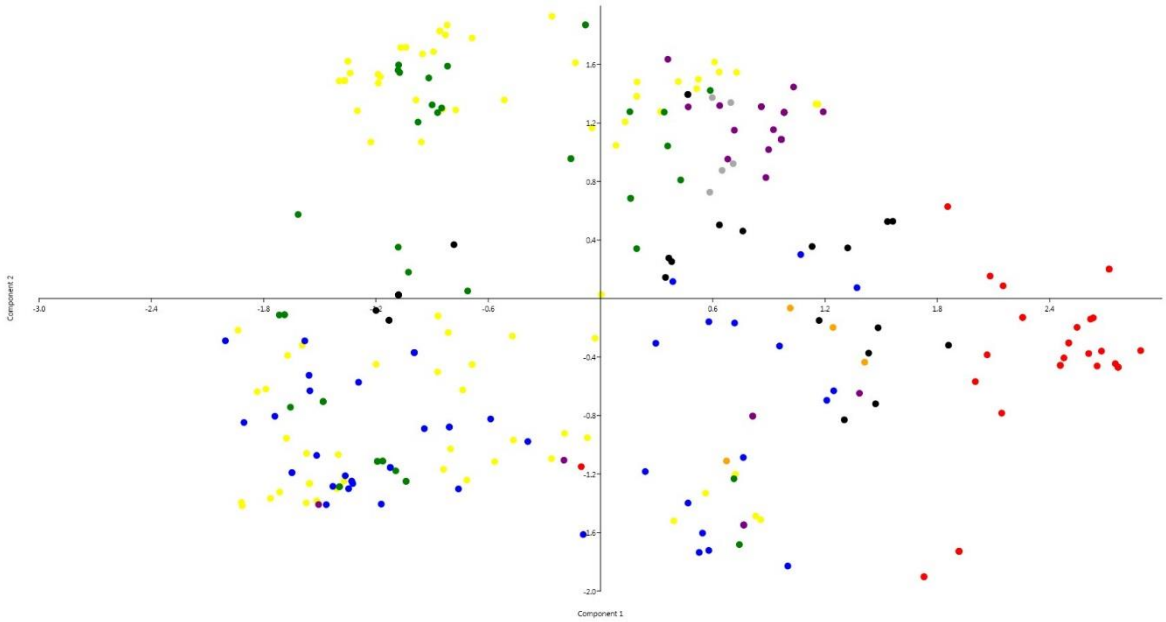


Figure 3.2. Virulence factors (VFs) distribution among different phylogroups using variance-covariance Principal Component Analysis (PCA). Phylogroups are represented as A = yellow, B1 = blue, B2 = red, C = orange, D = green, E = purple, F = black, clade I = grey. On average, phylogroup B2 strains had highest number of VFs and phylogroup B1 had the lowest.

3.4.4 *Resistance profile*

Overall, phylogroup F had the highest average of 2.9 resistance determinants, closely followed by phylogroup C with 2.2 and phylogroup E with 2.1. Phylogroup A had an average of 2, followed by B1 with an overall average of 1.6 resistance genes. Phylogroup B2 had the lowest average determinants among the major phylogroups of 0.8, phylogroup D had an average of 1, and clade I as the overall lowest with 0.6. Resistance genes with highest prevalence which were detected in all phylogroups were *blaTEM-1B* (35.4%), *tetA* (33.7%) and *sul2* (29.8%). *dfrA5* (27.6%) was also detected in high proportions and present in all phylogroups except B2 and clade I. Phylogroup B2-ST95 and F-ST354 strains were most likely to harbour *tetB* gene determinant among other genes conferring resistance to tetracycline. Also, *dfA17* was over-represented in F-ST354 strains (77.8%) while *dfrA5* was over-represented in phylogroup A-ST6053 strains (100%). Sulfonamide resistance initiated by *sul2* gene was clearly over-represented in E-ST57 strains (66.7%) and also to a lesser proportion in F-ST117 strains (41.2%), while A-ST6053 carried both *sul1* and *sul2* resistance determinants.

Among phylogroup A lineages, 68.4% of CC10 isolates exhibited resistance out of which 53.8% had MDR status, with an average of 1.74 resistance determinants (Excel worksheet 3.1). CC48 had an average of 2.2, with all but 1 strain (91.7%) harbouring resistance determinants and 63.6% of these strains were MDR. Out of 10 CC93 isolates, 60% carried resistance determinants where 66.7% were classified as MDR (two each of ST93 and ST3333). On the other hand, 47.1% of ST665 strains (also of CC665) exhibited resistance, with all strains MDR and the average number of resistance was 2. ST6053 strains were all MDR with an average of 6.1 resistance determinants (detail in Discussion section).

In phylogroup B1, CC86 had 1.25 average number of resistance determinants with 75% of strains exhibiting resistance and one strain MDR (Excel worksheet 3.1). CC155 strains with five different STs exhibited high resistance where 90.5% harboured resistance markers and an average of 2.5, and out of these resistant strains 84.2% were MDR. ST101 had an average of 1.25 with all strains harbouring resistance determinants like *tetA* (50%), *tetC* (25%) and *dfrA5* (50%). On the other hand, 40% of ST212 had resistance with average 0.8, both strains harbouring *blaTEM-1C* and *tetA*. Two out of three ST295 strains were MDR (average=2.3). One ST345 strain was MDR and also 1 ST602 (CC446) strain had *gyrA* mutation conferring quinolone resistance. Overall, *tetA* was the resistance gene that was overrepresented in strains of phylogroup B1 (41.7%).

In phylogroup B2, ST95 had an average of 0.9 resistance determinant, where all but 2 strains had *tetB*. On the other hand, all ST131 harboured resistance with an average of 2, making it the most resistant ST among phylogroup B2. ST135 had a single strain with resistance gene (*sul2*), and no resistance determinants were detected for ST355 and ST372 strains (Excel worksheet 3.1). Phylogroup D lineages, namely CC38 had 1 strain exhibiting resistance (average 0.4) while CC115 had an average of 0.7 with 3 strains exhibiting quinolone resistance (*gyrA* mutation) and 1 strain MDR with additional *tetB* and *sul2* genes. All (ST349) but 1 (ST6066) CC349 strains exhibited resistance with average of 1.4 determinants. ST68 had an average of 4 with all strains harbouring *blaTEM-1B*, *tetB*, *dfrA5* and *dfrA17*, making it the most resistant ST among phylogroup

D. ST69 had 0.4 with only 2 strains harbouring resistance markers, while strains of STs 1158, 362, 714 had 1 resistance gene each. ST1166 strains and ST405 strain did not harbour any resistance determinants (Excel worksheet 3.1).

In phylogroup E, CC350 had an average of 2.3 resistance markers, where all but 1 ST57 strain (95.6%) showed resistance and 78.3% were MDR (Excel worksheet 3.1). Resistance was seen to antimicrobials of classes beta-lactam (13.6% *blaTEM-1B*), tetracycline (27.3% *tetA*, 4.5% *tetB*, and 50% *tetC*), sulphonamide (13.6% *sul1*, 59.1% *sul2*), trimethoprim (18.2% *dfrA5*), aminoglycoside (45.4% *aadA1*) and also quinolone (13.6% *gyrA* mutation). ST219 with 2 strains had an average of 1.5, with both strains harbouring *tetA* and 1 strain also had *blaTEM-1B*. Single strain ST5281 had *tetA* while ST1011 had no resistance. Between the 2 phylogroup F lineages, ST354 had 4.5 and ST117 had 2 average resistance determinants. All ST354 strains had quinolone and fluoroquinolone resistance with *parC* mutation, while *gyrA* mutation (11.8%) was detected in 2 strains of ST117. The *gyrA* mutation confers resistance to quinolone but not fluoroquinolone.

All ST88 (CC23) strains of phylogroup C had resistance determinants with an average of 2.25. On the other hand, only 1 ST770 strain of clade I had resistance determinants (average 0.6), namely *blaTEM-1B*, *tetA* and *sul2* (i.e. 20%) (Excel worksheet 3.1).

With respect to the source of poultry meat, conventionally reared meat samples (n=245 strains) had the highest resistance gene determinants content with 65.7% (n=161 strains), then followed by free range poultry meat (n=11 strains) with 54.5% strains (n=6) exhibiting resistance (i.e. harbouring at least one resistance gene). Organic poultry meat *E. coli* strains (n=27) harboured the lowest number of resistance genes with 13 strains exhibiting resistance (48.1%).

Table 3.2. Plasmid-mediated and chromosomal-mediated resistance determinants by phylogroup distribution.

Resistance gene	Function	All strains %	A (n=92) %	B1 (n=48) %	B2 (n=37) %	C (n=4) %	D (n=43) %	E (n=28) %	F (n=26) %	Clade I (n=5) %
tetA	Tetracycline resistance	21.5	25	41.7	5.4	50	4.6	32.1	7.7	20
tetB	Tetracycline resistance	14.1	1.1	12.5	40.5	-	18.6	3.6	34.6	-
tetC	Tetracycline resistance	6.4	4.3	2.1	-	-	2.3	39.3	3.8	-
blaTEM-1A	Beta-lactam resistance	0.7	2.2	-	-	-	-	-	-	-
blaTEM-1B	Beta-lactam resistance	22.6	32.6	18.7	16.2	25	11.6	14.3	30.8	20
blaTEM-1C	Beta-lactam resistance	3.9	3.3	8.3	-	25	6.9	-	-	-
blaTEM-116	Beta-lactam resistance	0.7	1.1	-	-	25	-	-	-	-
blaCMY-2	Beta-lactam resistance	0.3	-	-	-	-	2.3	-	7.6	-
sul1	Sulfonamide resistance	8.5	16.3	-	2.7	-	4.6	10.7	11.5	-
sul2	Sulfonamide resistance	19.1	17.4	6.2	2.7	25	20.9	50	34.6	20
dfrA1	Trimethoprim resistance	1.6	4.3	-	-	-	-	-	-	-
dfrA5	Trimethoprim resistance	17.7	25	20.8	-	25	3.5	14.3	23.1	-
dfrA12	Trimethoprim resistance	0.7	-	-	5.4	-	-	-	-	-
dfrA14	Trimethoprim resistance	0.3	1.1	-	-	-	-	-	-	-
dfrA17	Trimethoprim resistance	3.2	1.1	2.1	-	-	-	-	26.9	-
strA	Aminoglycoside resistance	14.1	26.1	18.7	-	25	2.3	-	19.2	-
strB	Aminoglycoside resistance	14.1	23.9	22.9	-	25	0.6	-	19.2	-
aadA1	Aminoglycoside resistance	6.4	5.4	-	-	-	6.9	35.7	-	-
aadA2	Aminoglycoside resistance	0.3	-	-	2.7	-	-	-	-	-
aadA5	Aminoglycoside resistance	0.7	1.1	2.1	-	-	-	-	-	-
catA1	Phenicol resistance	1.4	-	6.2	-	-	-	-	3.8	-
aac(3)-IId	Aminoglycoside resistance	2.1	-	-	-	-	-	-	23.1	-
aph(3')-Ic	Aminoglycoside resistance	0.7	2.2	-	-	-	-	-	-	-
gyrA, parC mutation	Quinolone resistance	7.4	3.3	2.1	-	-	6.9	10.7	42.3	-

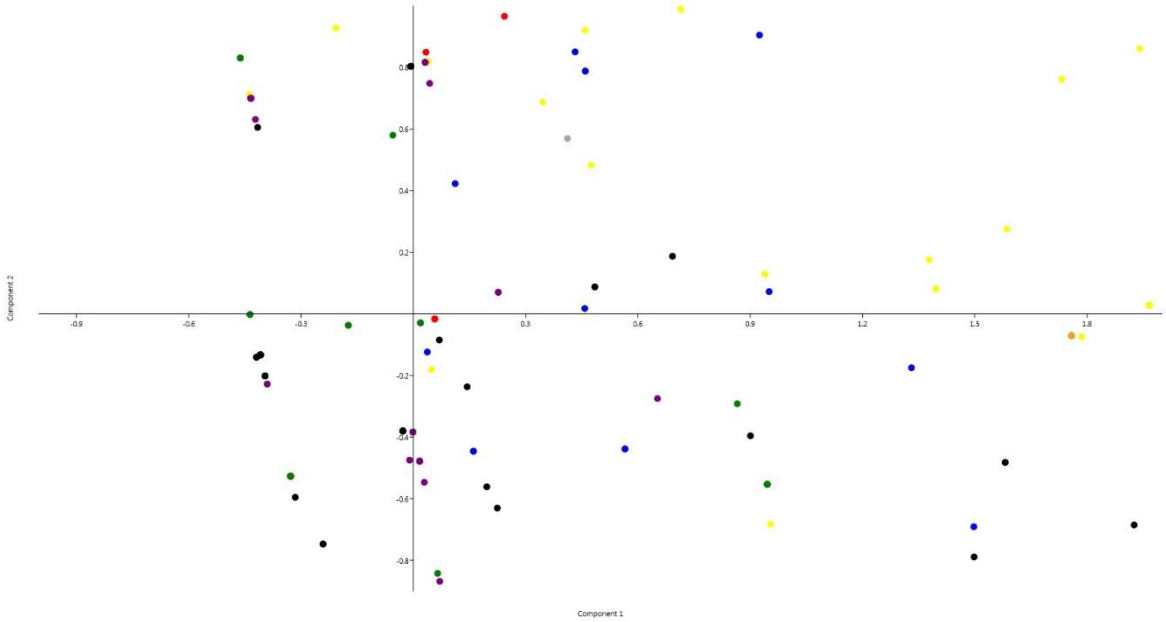


Figure 3.3. Plasmid-mediated resistance determinants distribution by phylogroup using variance-covariance. Principal Component Analysis (PCA). Phylogroups are represented as A = yellow, B1 = blue, B2 = red, C = orange, D = green, E = purple, F = black, clade I = grey. ST6053 of phylogroup A had highest average number of resistance (6.1) while ST135 of phylogroup B2 had the lowest (0.1).

3.4.5 *Incompatibility (Inc) groups distribution*

Out of 283 strains, 229 strains (80.9%) harboured one or more plasmid incompatibility (Inc) group or also referred as plasmid replicon type. In phylogroup A, 63 strains (68.5%) out of 92 harboured Inc group, IncFII was over-represented with 46 strains (73%) harbouring it, followed by IncFIB with 38 strains (60.3%) and IncI1 with 28 strains (44.4%). In phylogroup B1, 44 strains (91.7%) had one or more Inc group with IncFII over-represented (95.4%) followed by IncFIB (65.9%). All but one phylogroup B2 strains had Inc groups, all of these strains had IncFII, and all ST131 strains additionally had IncI1, IncFIB and IncFIC. Three ST135 and both ST355 strains had IncFIB and IncFIC additionally. All phylogroup C and clade I strains had IncFII and IncFIB.

In phylogroup D, the presence of Inc groups was relatively lower compared with other phylogroups (55.85). IncFII was present in all strains and IncFIB in 66.7% of strains, majority of which belonged to CC115 and CC349. On the other hand, all strains of

phylogroup E had Inc groups, with IncFII present in all strains, IncFIB in 25 strains (89.3%) and IncFIC in 21 strains (75%).

IncFIB was present in all but one phylogroup F strain of ST354. There was a clear distinction in Inc group content between the 2 STs 117 and 354 of this phylogroup, with IncFII present in 15 out of 17 (88.2%) ST117 strains, while IncFII was absent in all ST354 strains. Instead, IncFIA was present in all but one (88.9%) ST354 strains.

A distinct association of IncFIC and CC350 (STs 57 and 371) of phylogroup E was evident with 87.5% of the strains harbouring the Inc group. Also, IncFIA was over-represented in ST354 (phylogroup F) strains, but absent in strains of phylogroups B2, C, E and clade I, and even though present, were clearly under-represented in phylogroups A (9.5%), B1 (9.1%) and D (16.7%).

3.5 Discussion

In this study, a diverse genetic heterogeneity of *E. coli* isolated from poultry meat was identified, using whole genome sequencing approach. Out of 283 strains belonging to 8 different phylogroups, namely A, B1, B2, C, D, E, F and cryptic clade I, 69 different Achtman scheme STs were identified and 3 were un-typeable. Molecular serotyping with O and H-antigen typing yielded highly diverse results (61 O types with 111 strains un-typeable, 34 H types with one un-typeable, 92 O and H combinations), although serotyping has been indicated to not be as informative for specific lineage identification as MLST, with over 173 O types and 56 different H types present (Fratamico et al., 2016). The ST distribution of poultry meat *E. coli* strains differ quite distinctly from human strains STs (Gordon DM, unpublished data), with relatively little human like *E. coli* present in poultry meat based on their ST. For instance, STs 59, 648 of phylogroup F were completely absent in poultry strains but made up 51.7% in humans (Appendix; Supplemental Table 3.1 & 3.2). On the other hand, poultry-associated STs like ST117, ST57 were over-represented in poultry meat but very low (ST117, Chapter 4) or absent (ST57) in human isolates. This data indicates the prevalence of poultry associated STs in

poultry meat that are rarely observed in humans even though the samples are both from the same region. One ST can belong to many different serotypes as with the cases of STs like 10, 48, 57, 69, 117.

Phylogroup A strains were over represented (32.5%), which was consistent with other findings, where the phylogroup was typically highly represented in broilers or poultry in general (Johnson et al., 2003, Obeng et al., 2012, Pasquali et al., 2015). The phylogroup has also often been associated with intestinal infections worldwide and to a lesser extent ExPEC, especially STs 10 and 48 (Manges and Johnson, 2012, Clermont et al., 2011). Phylogroup A was followed by B1 (17%), another group commonly associated as a commensal, also known to have broad host spectrum with its sister group i.e. phylogroup A (Touchon et al., 2009, Tenaillon et al., 2010). Phylogroup B1 were found largely in secondary habitats of *E. coli* i.e. in environmental samples like water (Gordon and Cowling, 2003). Out of the four major phylogroups, phylogroups D and B2 were present in lesser proportions (15.2% and 13.1% respectively), the phylogroups known to be more host-specific with narrow host spectrum, and often associated with ExPEC infections (Picard et al., 1999, Johnson et al., 2001). Among the minor phylogroups, phylogroup E (commonly associated with intestinal infections) was present at the highest frequency (9.9%), followed by phylogroup F (9.2%) also known as a sister group of B2, cryptic clade I (1.8%), a lineage phenotypically identical to *E. coli*, and then phylogroup C (1.4%), which is closely related to B1 (Clermont et al., 2011, 2013).

Among phylogroup A, CC10 and CC48 strains made up 33.7% of all the A strains, and were closely related where STs 48, 1137 and 2470 had one allele difference, while STs 2207, 2739, 6050 and 6051 had two allele differences to ST10. Two ST10 strains harboured VFs commonly associated with enteropathogenic *E. coli* (EPEC) also known as diarrheal *E. coli* like *tir*, *nleB*, *espA, espB*, *cif* and *eae* (Kaper et al., 2004), and had the highest number of VFs out of the closely related lineages CC10 and CC48 strains. ST3333 had one allele difference from ST93 along with STs 373, 3770, 6061 (all classified as CC93). The strains were *arpA*, *yjaA* and *tsp* positive, which does not cluster with any of the phylogroups defined in the new Clermont phylogrouping method (Clermont et al.,

2013). This could indicate that the strains of this lineage gained *tsp* gene, as gene gain is a common phenomenon in *E. coli* (Touchon et al., 2009). The strains were MDR and harboured five plasmid-mediated and one chromosomal resistance determinants conferring resistance to six classes of antimicrobials (*blaTEM-1C, tetA, sul1, dfrA1, aadA1* and *gyrA* mutation).

Also in phylogroup A, STs 665, 6047 and 6053 were the over-represented STs, out of which 2 STs were novel i.e. ST6047 and ST6053. These STs were observed to harbour higher average number of VFs (majority of which were ExPEC related genes) as compared to other widely studied STs of phylogroup A, like ST10 and ST48 which are often associated with intestinal and also extra-intestinal infections (Manges and Johnson, 2012). ST665 had two different clusters, 1 with phylogroup A0 profile i.e. only *arpA* positive, and the other gave a phylogroup D1 profile i.e. *arpA* and *chuA* positive. Of note, all 6 strains with D1-ST665 profile had *ireA* gene, which was absent in the remaining eleven strains with A0-ST665 profile. The other 2 STs 6047 and 6053 were also *arpA* and *chuA* positive, also classified as phylogroup D using the new Clermont method. This could indicate recombination between two phylogroups, where the strains gained the gene *chuA* from the latter. ST6053 strains were all MDR, with 6 resistance determinants (*blaTEM-1B, sul1, sul2, dfrA5, strA* and *strB*) and 1 strain with 7 determinants (additional *tetA* gene). In addition, APEC-specific genes were detected in 12% of phylogroup A strains (CC665=5, ST6053=3 and CC93=3), while they were absent in the remaining clonal groups of phylogroup A, indicating clonal specificity. ST399 had the least VFs and none of the strains had resistance determinants. Overall, 55.4% of the strains exhibited resistance, with 72.5% of these strains MDR, *blaTEM-1B* was the most prevalent followed by *strA*. One ST10 and ST373 each had ExPEC status/ trait, and ST10 strain was coupled with MDR.

Phylogroup B1 had diverse STs, out of which STs 101, 212, 297, 345 and 602 harboured higher numbers of VFs often associated with ExPEC (*iss, iroN, omp, upaG, sitA, traT*) and also had *lpfA_LF82* variant, compared to over-represented STs like ST58 and ST155 of CC155. All ST101 strains also exhibited resistance, highlighting that this clonal group

with potential virulence is also resistant consistent with findings by Mora et al., 2011. On the other hand, CC155 had the highest average resistance determinants of 2.5, with ST155 having 2.9 followed by ST58 with 2.5. A strain of ST155 had 8 resistance markers (*blaTEM-1B, tetB, sul2, dfrA17, strA, strB, aadA5, catA1*), the second highest among all the strains in this study and MDR was seen in 76.2% of CC155 strains. Also noteworthy was that all ST58 (PST87 of Pasteur scheme CC87) strains were MDR; the clonal complex was described as having animal origin, and linked to increased AMR prevalence in the environment in a study by Skurnik et al., 2016. Overall, phylogroup B1 had the lowest average number of VFs (14.3) among the 8 phylogroups identified, and 70.8% exhibit resistance (out of which 67.6% were MDR), with average resistance determinants of 1.6, lower than A but higher than B2 and D's. Two strains of ST58 (also MDR) and ST1841 had ExPEC status harbouring both *iutA* and *papC* genes. Furthermore, 6 strains of phylogroup B1 (12.5%) harboured APEC-specific genes, 3 belonging to CC155 (ST58=1, ST155=2), and 1 strain each of STs 345, 1125, 1841.

Phylogroup B2 was more homogeneous in distribution compared with other major phylogroups, with 3 main STs 95, 131 and 135 and 2 under-represented STs 355 and 372. Similar to other studies on *E. coli* in poultry meat (Aslam et al., 2014, Manges et al., 2015), phylogroup B2 harboured higher average number of VFs compared to other phylogroups, with 48.6% and 70.3% of strains having ExPEC and APEC traits respectively. APEC and ExPEC lineages (Mora et al., 2009, 2011, Johnson et al., 2007) like ST95 followed by ST131, consistently harboured the highest average number of virulence determinants (30.2 and 29.5 respectively). ST135 strains made up 29.7% of all B2 isolates, the lineage was linked to APEC causing septicemia in poultry birds and also belonged to O2:H1 serotype, according to MLST database http://mlst.warwick.ac.uk/mlst/dbs/Ecoli (accessed on 19th September, 2016). The ST135 strains harboured ExPEC associated VFs like *etsC*, *fimH*, *fyuA*, *ibeA*, *iroN*, *kpsE*, *ompT*, *sitA*, *tratT*, *usp*, *vat*, ybtS, *upaG*, *arcA*, *iss*, *gad*, *mchF*, *cva,* and *cia*. ST355 strains (n=2), though under-represented also harboured high number of VFs (30 VFs each) similar to STs 95 and 131, and were also associated with APEC, O2:H5 serotype, as per MLST database. Based on the prevalence of APEC-specific genes in certain lineages of

phylogroup B2 (STs 95, 131, 135, 355), this can be linked to clonal specificity and is apparent in this phylogroup.

In this study, 59.5% of B2 strains harboured at least one plasmid-mediated resistance determinants. Only 2 strains could be classified as MDR (both ST131), the phylogroup had evidently lower levels of resistance (with an average of 0.76), the lowest among the major phylogroups. This finding is consistent with other studies on phylogroup B2 in humans and poultry, which is associated to have lower resistance and higher virulence (Obeng et al., 2012), except for ST131 which are often highly virulent with MDR status (Mora et al., 2010, Rogers et al., 2011, Banerjee et al., 2013).

Two strains of phylogroup C harboured ExPEC and APEC-specific genes and one strain was also MDR. All the strains belonged to ST88, a member of ST23 clonal complex (CC23) which has been associated with ExPEC and APEC infections (Maluta et al., 2014a). All of the strains had plasmid-mediated resistance with two of them MDR with an average of 2.25 resistance determinants. ExPEC related VFs like *iroN, omp, sitA, traT, upaG, mchF* and also ETEC related gene *lpfA* (Maluta et al., 2014b) were present in all the strains thus further establishing the virulence potential of ST88 (CC23) lineage. Among the clade I (ST770) strains, only one strain exhibited MDR to five antimicrobial classes while the other four strains lacked resistance determinants. On the other hand, all strains had ExPEC status and had high average number of VFs (24.6), commonly to ExPEC associated genes like *iss, iucC, kpsE, omp, sitA, traT, fyuA, etsC, ybtS, iha* (Köhler and Dobrindt, 2011) and also bacteriocins (*colicin Ia* and *Ib*). The genes tia and *hra* were also present in all strains, which are invasin and adhesin genes commonly associated with EAEC (Mancini et al., 2011).

In phylogroup D, ST69 and CC38 (both 20.9%) made up the majority of strains in phylogroup D where ST69 is a member of the widely studied uropathogenic related clonal group A or CGA (Manges et al., 2001). The strains did not exhibit resistance except for 1 strain in CC38 and two strains in ST69 unlike other studies where CGA had been associated to exhibit MDR (Manges et al., 2001). ST1166 were initially assigned to

phylogroup E, as they were *yjaA* positive, which did not correspond to profiles of phylogroup D sub-groups i.e. D1 nor D2. Most likely, these strains gained *yjaA* through gene transfer. Overall, 46.5% of the strains had resistance determinants, with 55% out of these exhibiting MDR i.e. over 25.6% of the total phylogroup D strains, where ST68 had the highest average resistance (4). Nine strains (20.9%) had ExPEC status (ST115=4, ST349=2, ST1158=2, ST714=1), out of which 3 were also MDR (ST349=2, ST115=1), while 3 strains had APEC traits (ST69=2, ST362=1). ST1158 had the highest average VFs (23) among all the phylogroup D strains followed by CC115 (21.4), ST362 (21), ST69 (19.5) and CC349 (18.4) strains, majority of which were ExPEC-related genes namely, *fimH*, *kpsE*, *sitA*, *cah*, *iha*, *eaeH*, *upaG*. Noteworthy, all ST115 strains exhibited ExPEC trait with 3 strains (75%) also demonstrating quinolone (nalidixic acid) resistance, which highlights the significance of this poultry lineage ST (Cortés et al., 2010) as a potential ExPEC/ APEC lineage, coupled with resistance to an antimicrobial class (quinolone) critically important to humans.

In phylogroup E, ST57 of CC350 was clearly predominant, and harboured a relatively large number of virulence determinants which could indicate that this ST is a poultry-associated lineage with potential zoonosis for humans and may also be capable of causing infections in birds. The over-represented VFs were mostly ExPEC associated genes like *iss, iucC, iutA, sitA, papC, kpsE*, *cma*. ST57 was associated with poultry source (both from APEC in Germany), except in one case (ECOR31) where it was isolated from leopard in United States (accessed on 7th September, 2016). Additionally in this study, APEC-specific genes were present in 87.5% of CC350 strains clearly associating this clonal group to APEC lineage. O115:H25 (n=8) was the most prevalent serotype with ST57 isolates (n=21) belonging to 9 different serotypes, indicating that certain clonal lineages have highly variable serotype diversity. Overall, 70.4% of phylogroup E strains were MDR and all strains harboured IncFII. This highlights that CC350, besides being potentially zoonotic with high VF content, are also highly resistant.

Phylogroup F had two distinct STs, ST117 and ST354. Detailed studies on these STs and genetic comparison with strains from different sources of same STs were presented in

the following chapter 4 and paper (Vangchhia et al, 2016) respectively. 64.7% of ST117 strains had APEC-specific genes potentially linking this clonal group to APEC lineage as supported by other studies (Cordoni et al., 2016, Dissanayake et al., 2014, Mora et al., 2012).

Overall, 64% of the strains harboured resistance gene determinants out of which 61.3% were classified as MDR with resistance to 2 or more antimicrobial classes. The highest number of resistance determinants in a strain was 9, belonging to F-ST354 having *blaTEM-1B*, *tetB*, *sul2*, *strA*, *strB*, *dfrA5*, *dfrA17*, *aac(3)-IId* and *parC* mutation. All 283 strains harboured virulence factors ranging from a minimum of 3 VFs (A-ST399) to a maximum of 32 VFs (B2-ST95). Out of the strains that harboured more VFs, F-ST117 had the highest proportion of MDR in a clonal group (41.2%), which further adds the significance of this ST as a potential pathogen to humans (Mora et al., 2012). Overall, 19.4% (n=55) of the total strains had ExPEC status i.e. having two or more of *sfa/foc*, *afa/dra*, *papA/C*, *kpsMT* II or *iutA* as per Johnson et al., 2003. A distinct association of phylogroups to ExPEC trait prevalence was evident, with the highest in phylogroup B2 (32.7%), followed by E (25.4%), D (16.4%), Clade I (9.1%), B1 (5.4%), then A, C and F (3.6% each). Out of these strains with potential ExPEC trait, 38.2% (n=21) were MDR, 52.4% were E-ST57 strains (n=11), 2 each of F-ST117 and D-ST349, and 1 strain each of A-ST10, C-ST88, D-ST115, E-ST219 and Clade I-ST770. These findings of highly virulent strains coupled with plasmid-mediated MDR stresses the relevance of finding these strains in poultry meat destined for human consumption. For APEC specificity, it was evident that phylogroups E (75%) and B2 (70.3%) were more clearly associated with the APEC lineage as compared to the other phylogroups like A (12%), B1 (12.5%), and D (7%).

In this study, 22 strains (7.8%) were found to exhibit resistance to nalidixic acid (quinolone) out of which 9 strains were also resistant to ciprofloxacin (fluoroquinolone) phenotypically. All of these strains had *gyrA* (for strains resistant to only nalidixic acid) and *parC* (for strains resistant to both nalidixic acid and ciprofloxacin) chromosomal mutations, which are known to aid resistance to quinolone (Johnson et al., 2003, Sanders, 2001). Our findings is in contrast to another Australian study on *E. coli* in

poultry meat conducted by Obeng et al., 2012 in South Australia, where they did not find any quinolone resistance. In Australia, the use of quinolones has never been approved in food-producing animals (Cheng et al., 2012). The presence of quinolone resistant isolates was low (12.1%) among isolates exhibiting resistance compared to other countries like United States (37%) (Johnson et al., 2003), though this does not rule out its relevance. This could indicate cross-contamination i.e. entry of resistant whole bacterium isolates' from other sources (say, humans) as the use of this antimicrobial class is not restricted in humans.

Another possible occurrence suggested by Ingram et al., 2013 is co-selection, as the strains were also resistant to antimicrobials from other classes namely beta-lactams (ampicillin), aminoglycosides (streptomycin), and sulfonamide (sulfamethoxazole). However, whole bacterium vertical transfer and successful circulation is more likely as the resistance is mediated through chromosomal mutation. To the best of our knowledge, till date there have not been any reports of plasmid-mediated colistin resistance in *E. coli*, a last line drug class after emergence of carbapenem resistance. This elicited a worldwide concern when it was first reported in China (Liu et al, 2015), followed by a number of reports from other countries including developed countries like Netherlands (Kluytmans et al., 2016), United Kingdom (Doumith et al., 2016), United States (Meinersmann et al., 2016), Denmark (Hasman et al., 2015). The presence of resistance gene determinants to widely used antimicrobial classes in the poultry industry like penicillin, tetracycline and sulfonamide pose as a threat not only to human health but maybe even more so to the poultry industry itself. These antibiotics will soon prove to be ineffective in treating or preventing outbreak of infections, if they continue to be used as widely as they are now, thus adversely leading to the need of using broad-spectrum antibiotics. Therefore, the implementation of strict policies in antimicrobial use and continued surveillance is crucial in curbing antimicrobial resistance in both public health and livestock sectors (JETACAR, 1999).

This study contributes to the wide array of genetic information available on *E. coli* in poultry meat, using whole genome sequences rather than using different molecular

typing procedures for MLST, serotyping, virulence and resistance determinants typing. An extensive review on *E. coli* in food-producing animals by Lazarus et al., 2015 had pointed that whole genome sequencing (WGS) would be the ideal method to streamline genetic studies worldwide. WGS makes it more accessible for researchers to compare findings as whole genome data can be easily deposited to online databases like NCBI, Enterobase, PubMLST to name a few. Though there are a number of studies which have focused specifically on virulence factors and antimicrobial resistance determinants (Manges et al, 2015, Kluytmans et al., 2013, Johnson et al., 2009), limited studies are available using whole genome-based approach. Unlike this study which has both clinically relevant and commensal populations of *E. coli*, a number of genetic studies tend to be biased and more focussed on only clinically relevant strains (Leimbach et al., 2013). This poses a huge limitation in genetic diversity studies of organisms like *E. coli* which are highly heterogeneous and highlights the importance of selecting phylogenetically diverse strains for sequencing to determine substantial phylogenetic ancestry or lineage.

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3.7 Supplemental Materials

Supplemental Table 3.1. Phylogroup, serotype/ serogroup, ST distribution of all 283 poultry meat strains

Strain	ST	CC	Phylogroup	O type	H type	Serotype
A3.23	10	10	A	O182	H19	O182:H19
A3.61	10	10	A	O148	H32	O148:H32
A3.62	10	10	A		H32	:H32
A3.67	10	10	A	O182	H19	O182:H19
A3.74	10	10	A	O132	H21	O132:H21
A4.15	10	10	A	O127	H21	O127:H21
A4.33	10	10	A	O69	H11	O69:H11
A4.49	10	10	A	O6	H2	O6:H2
A4.50	10	10	A	O182	H19	O182:H19
C1.10	10	10	A	O49	H32	O49:H32
C3.12	10	10	A		H32	:H32
C3.13	10	10	A	O16	H48	O16:H48
C3.14	10	10	A		H21	:H21
C3.6	10	10	A	O6	H2	O6:H2
C3.9	10	10	A	O177	H32	O177:H32
C4.17	10	10	A		H32	:H32
C1.33	1137	10	A	O23	H48	O23:H48
C1.36	2207	10	A	O117	H42	O117:H42
C3.15	2470	10	A	O132	H21	O132:H21
A2.3	48	48	A	O15	H11	O15:H11
A2.35	48	48	A		H11	:H11
A2.41	48	48	A	O26	H12	O26:H12
A4.11	48	48	A	O26	H12	O26:H12
C1.15	48	48	A	O86	H16	O86:H16
C1.30	48	48	A	O8	H11	O8:H11
C2.19	48	48	A	OgC4/O118/O151		OgC4/O118/O151:
C2.8	48	48	A		H16	:H16
C2.12	2739	48	A	O25	H16	O25:H16
C2.18	2739	48	A	O25	H16	O25:H16

A1.25	6050	48	A		H45	:H45
A1.28a	6051	48	A		H11	:H11
A3.22	93	93	A	O7	H4	O7:H4
C3.2	93	93	A	O6	H16	O6:H16
C3.3	93	93	A		H16	:H16
W2.16	93	93	A		H4	:H4
A2.58	373	93	A	O21	H16	O21:H16
W2.9	6061	93	A	O21	H52	O21:H52
W3.57	3333	93	A	O132	H52	O132:H52
W3.58	3333	93	A		H52	:H52
C2.16	3770	93	A		H10	:H10
W1.2	Unknown	93	A	O5	H10	O5:H10
A2.2	206	206	A	O96	H5	O96:H5
A2.47	206	206	A		H5	:H5
C3.10	206	206	A		H5	:H5
C3.11	206	206	A		H5	:H5
C4.2	216	216	A		H4	:H4
A1.1	399	399	A	O15	H12	O15:H12
A2.49	399	399	A		H12	:H12
C1.13	399	399	A		H12	:H12
C2.14	399	399	A	O13/O135	H30	O13/O135:H30
A1.33	665	665	A		H4	:H4
A2.46	665	665	A		H4	:H4
A2.8	665	665	A		H4	:H4
A3.31	665	665	A		H4	:H4
A3.70	665	665	A		H4	:H4
A4.27	665	665	A		H4	:H4
C1.38	665	665	A		H4	:H4
C3.39	665	665	A		H4	:H4
C3.40	665	665	A		H4	:H4
C3.5	665	665	A	O78	H4	O78:H4
C4.8	665	665	A		H4	:H4
W2.4	665	665	A	O78	H4	O78:H4
W3.2	665	665	A		H4	:H4
W3.63	665	665	A		H4	:H4
W4.1	665	665	A		H4	:H4
W4.40	665	665	A		H4	:H4
W4.8	665	665	A	O78	H4	O78:H4
A4.35	746	746	A	O9	H37	O9:H37
A3.18	1408	1408	A		H30	:H30
C2.5	2705	373	A		H10	:H10
C1.3	5295	5295	A	O71	H12	O71:H12
A1.20	6047	6047	A	O45	H19	O45:H19
A2.31	6047	6047	A		H4	:H4
A2.32	6047	6047	A		H4	:H4
A2.33	6047	6047	A		H4	:H4

A4.28	6047	6047	A		H4	:H4
C1.1	6047	6047	A		H4	:H4
C1.89	6047	6047	A		H4	:H4
C2.58	6047	6047	A		H4	:H4
C4.48	6047	6047	A		H4	:H4
W1.67	6047	6047	A		H4	:H4
W1.69	6047	6047	A		H4	:H4
W3.61	6047	6047	A		H4	:H4
W4.59	6047	6047	A		H4	:H4
A2.59	6053	6047	A		H4	:H4
C1.90	6053	6047	A		H4	:H4
C2.53	6053	6047	A	O11	H4	O11:H4
C4.52	6053	6047	A	O11	H4	O11:H4
W1.66	6053	6047	A		H4	:H4
W2.78	6053	6047	A	O11	H4	O11:H4
W4.48	6053	6047	A	O11	H4	O11:H4
W4.68	6053	6047	A	O11	H4	O11:H4
C3.19	453	86	B1	O23	H16	O23:H16
C4.24	453	86	B1		H16	:H16
A3.64	641	86	B1		H21	:H21
C1.61	641	86	B1	O159	H21	O159:H21
A4.14	101	101	B1		H8	:H8
C4.21	101	101	B1	O82	H8	O82:H8
W1.32	101	101	B1	O16	H8	O16:H8
W4.20	101	101	B1	O82	H8	O82:H8
A1.38	58	155	B1		H37	:H37
A2.36	58	155	B1		H37	:H37
A3.41	58	155	B1		H37	:H37
C1.66	58	155	B1		H37	:H37
W3.17	58	155	B1	O11	H25	O11:H25
W3.24	58	155	B1		H37	:H37
W4.22	58	155	B1		H37	:H37
A1.44	155	155	B1	O9	H10	O9:H10
C1.72	155	155	B1		H4	:H4
C1.84	155	155	B1		H51	:H51
W1.33	155	155	B1	O8	H51	O8:H51
W2.37	155	155	B1	O86	H51	O86:H51
W2.41	155	155	B1	O180	H9	O180:H9
W3.32	155	155	B1	O5	H11	O5:H11
W4.17	155	155	B1	O8	H51	O8:H51
C2.34	683	155	B1	O100	H25	O100:H25
W1.37	683	155	B1	O100	H25	O100:H25
W3.20	683	155	B1	O100	H25	O100:H25
C1.51	949	155	B1	O29	H9	O29:H9
A2.17	6062	155	B1	O29	H9	O29:H9
A2.19	6062	155	B1	O29	H9	O29:H9

A2.40	212	212	B1	O18ac	H49	O18ac:H49
A2.5	212	212	B1	O18	H49	O18:H49
A3.17	212	212	B1	O18ac	H49	O18ac:H49
A4.37	212	212	B1	O18ac	H49	O18ac:H49
C3.22	212	212	B1	O18ac	H49	O18ac:H49
C1.79	224	224	B1		H23	:H23
A3.45	906	906	B1	O150	H8	O150:H8
C2.38	295	295	B1		H16	:H16
C4.19	295	295	B1		H16	:H16
W2.31	295	295	B1	O185	H16	O185:H16
A4.3	297	297	B1	O45	H8	O45:H8
A4.57	345	345	B1	O8	H21	O8:H21
C2.41	345	345	B1	O160	H11	O160:H11
A1.31	602	602	B1		H21	:H21
W4.30	602	602	B1		H21	:H21
W1.36	1125	1125	B1	O139	H19	O139:H19
C4.97	1841	1841	B1	O103	H7	O103:H7
W2.28	3190	3190	B1		H10	:H10
C2.22	6046	6046	B1	O173	H37	O173:H37
A1.66	95	95	B2	O1	H7	O1:H7
C2.110	95	95	B2		H7	:H7
C2.117	95	95	B2		H7	:H7
C3.27	95	95	B2	O1	H7	O1:H7
C3.28	95	95	B2		H7	:H7
C4.33	95	95	B2		H7	:H7
C4.34	95	95	B2	O1	H7	O1:H7
C4.35	95	95	B2		H7	:H7
W1.58	95	95	B2	O1	H7	O1:H7
W2.47	95	95	B2	O1	H7	O1:H7
W2.49	95	95	B2		H7	:H7
W2.53	95	95	B2	O1	H7	O1:H7
W4.32	95	95	B2	O1	H7	O1:H7
W4.33	95	95	B2	O1	H7	O1:H7
W4.35	95	95	B2		H7	:H7
W4.37	95	95	B2		H7	:H7
W4.39	95	95	B2	O1	H7	O1:H7
A1.49	131	131	B2	O25	H4	O25:H4
C2.116	131	131	B2	O25	H4	O25:H4
C3.29	131	131	B2	O25	H4	O25:H4
C4.100	131	131	B2	O25	H4	O25:H4
W1.57	131	131	B2	O25	H4	O25:H4
W1.65	131	131	B2	O25	H4	O25:H4
A1.30	135	135	B2	O2	H1	O2:H1
A3.5	135	135	B2	O50/O2	H1	O50/O2:H1
A4.5	135	135	B2	O2	H1	O2:H1
C1.46	135	135	B2	O50/O2	H1	O50/O2:H1

C3.30	135	135	B2	O50/O2	H1	O50/O2:H1
W2.50	135	135	B2	O50/O2	H1	O50/O2:H1
W2.51	135	135	B2	O50/O2	H1	O50/O2:H1
W2.56	135	135	B2	O2	H1	O2:H1
W3.35	135	135	B2	O2	H1	O2:H1
W4.34	135	135	B2	O50/O2	H1	O50/O2:H1
W4.36	135	135	B2	O2	H1	O2:H1
C4.32	355	355	B2	O50/O2	H5	O50/O2:H5
W3.33	355	355	B2	O50/O2	H5	O50/O2:H5
A2.15	372	372	B2	O83	H31	O83:H31
A3.16	88	88	C	O9	H12	O9:H12
C1.4	88	88	C	O9	H17	O9:H17
C3.8	88	88	C	O8	H9	O8:H9
A1.32	Unknown	88	C	O8	H9	O8:H9
A3.55	770	770	CladeI	O15	H16	O15:H16
C2.118	770	770	CladeI	O15	H16	O15:H16
C2.119	770	770	CladeI	O15	H16	O15:H16
C2.64	770	770	CladeI	O15	H16	O15:H16
W2.89	770	770	CladeI	O15	H16	O15:H16
A1.40	38	38	D		H10	:H10
A1.42	38	38	D	O7	H10	O7:H10
A1.45	38	38	D	O7	H10	O7:H10
A3.32	38	38	D		H9	:H9
A3.36	38	38	D		H9	:H9
A3.60	38	38	D		H10	:H10
W1.76	38	38	D		H15	:H15
C4.43	315	38	D		H15	:H15
C4.44	315	38	D		H15	:H15
A2.30	68	68	D		H6	:H6
A2.7	68	68	D	O1	H6	O1:H6
C2.93	68	68	D	O1	H6	O1:H6
C4.41	68	68	D	O1	H6	O1:H6
W2.75	68	68	D	O1	H6	O1:H6
A4.61	69	69	D		H49	:H49
C1.91	69	69	D	O17/O44	H18	O17/O44:H18
C2.45	69	69	D		H49	:H49
C4.42	69	69	D	O17/O44	H18	O17/O44:H18
W2.76	69	69	D	O17/O77	H18	O17/O77:H18
W2.79	69	69	D	O21	H18	O21:H18
W2.80	69	69	D		H49	:H49
W3.62	69	69	D	O17/O44	H18	O17/O44:H18
W3.67	69	69	D	O17/O77	H18	O17/O77:H18
A4.16	115	115	D	O2	H9	O2:H9
C1.95	115	115	D	O2	H6	O2:H6
C3.36	115	115	D		H9	:H9
C3.37	115	115	D	O2	H9	O2:H9

W2.77	2309	115	D	O15	H6	O15:H6
W3.59	2309	115	D		H6	:H6
W4.55	2309	115	D	O15	H6	O15:H6
C2.44	349	349	D	O49	H15	O49:H15
C2.46	349	349	D	O49	H15	O49:H15
C4.36	349	349	D		H15	:H15
C4.39	349	349	D		H15	:H15
A4.29	6066	349	D		H15	:H15
C3.59	362	362	D	O15	H1	O15:H1
A3.52	405	405	D	O50/O2	H6	O50/O2:H6
C4.38	714	714	D		H20	:H20
W2.83	1158	1158	D		H34	:H34
W3.73	1158	1158	D	O17/O44	H34	O17/O44:H34
W4.61	Unknown	1158	D	O25	H4	O25:H4
A3.11	1166	1166	D	O13	H31	O13:H31
A3.59	1166	1166	D	O13/O135	H31	O13/O135:H31
A3.48	219	219	E	O138	H48	O138:H48
C3.7	219	219	E		H48	:H48
A1.12	57	350	E	O109	H25	O109:H25
A2.22	57	350	E		H26	:H26
A3.57	57	350	E	O182	H25	O182:H25
A3.8	57	350	E		H25	:H25
A3.9	57	350	E	O115	H25	O115:H25
A4.12	57	350	E	O115	H25	O115:H25
A4.2	57	350	E		H25	:H25
A4.32	57	350	E	O115	H25	O115:H25
A4.9	57	350	E	O182	H25	O182:H25
C1.101	57	350	E	O182	H25	O182:H25
C1.102	57	350	E	O115	H25	O115:H25
C1.111	57	350	E	O128ac	H25	O128ac:H25
C2.115	57	350	E		H25	:H25
C2.68	57	350	E	O115	H25	O115:H25
C2.94	57	350	E	O115	H25	O115:H25
C3.43	57	350	E	O78	H25	O78:H25
C4.53	57	350	E	O115	H25	O115:H25
C4.65	57	350	E		H25	:H25
W1.79	57	350	E		H18	H18
W1.80	57	350	E		H18	H18
W3.72	57	350	E	O115	H25	O115:H25
A1.14	371	350	E	O45	H19	O45:H19
A1.29	371	350	E	O45	H19	O45:H19
C4.56	371	350	E	O45	H19	O45:H19
W2.87	1011	1011	E	O166	H45	O166:H45
C1.110	5281	5281	E		H28	:H28
A1.28	354	354	F		H34	:H34
A2.10	354	354	F		H34	:H34

A2.14	354	354	F		H34	:H34
A3.58	354	354	F		H34	:H34
C1.121	354	354	F		H34	:H34
C2.108	354	354	F		H34	:H34
C3.71	354	354	F		H34	:H34
C4.89	354	354	F	O3	H34	O3:H34
W2.68	354	354	F		H34	:H34
C1.119	117	117	H299	O45	H18	O45:H18
C1.123	117	117	H299	O161	H4	O161:H4
C2.100	117	117	H299	O111	H4	O111:H4
C2.102	117	117	H299	O111	H4	O111:H4
C2.103	117	117	H299	O119	H4	O119:H4
C2.106	117	117	H299	O71	H4	O71:H4
C2.13	117	117	H299		H4	:H4
C2.27	117	117	H299	O53	H4	O53:H4
C2.97	117	117	H299	O45	H4	O45:H4
C3.65	117	117	H299		H4	:H4
C3.70	117	117	H299	O45	H4	O45:H4
C3.72	117	117	H299	O149	H10	O149:H10
C4.85	117	117	H299	O143	H4	O143:H4
C4.86	117	117	H299	O53	H4	O53:H4
C4.93	117	117	H299	O71	H4	O71:H4
C4.94	117	117	H299	O45	H4	O45:H4
C4.95	117	117	H299	O45	H18	O45:H18

Supplemental Table 3.2. ST and phylogroup distribution of human strains

Strain	ST	Phylogroup
64-TC1	10	A
H197	10	A
H228	10	A
H288	10	A
H383	10	A
H386	10	A
H454	10	A
H617	10	A
H646	10	A
H730	10	A
2H_157_1	10	A
2H_189_7	10	A
2H_228_15	10	A
2H_277_1	10	A
2H_319_6	10	A
2H_343_1	10	A
64_3_AC10	10	A

65_2_AC1	10	A
8_2_Ti16_redo	10	A
H035	10	A
H287	10	A
H447	10	A
H474	10	A
H521	10	A
H668	10	A
H758	10	A
H778	12003	A
3_1_AU1	1316	A
H025	1434	A
H389	1711	A
H483	1917	A
H451	206	A
H185	216	A
H499	216	A
16_1_Ti5	216	A
67_4_Ti7	216	A
H572	216	A
H736	227	A
H562	227	A
H645	227	A
7_14_10	2514	A
CD_59_LN	2606	A
H173	2731	A
H315	2795	A
H472	2795	A
H019	2967	A
H482	2967	A
H484	2967	A
H158	34	A
5_AD	3478	A
H440	3588	A
H549	3589	A
H053	3764	A
57-2-RSi1	398	A
45_2_HU10	398	A
45_7_HU9	398	A
H656	398	A
H143	399	A
57_2_DC4	409	A
CD_59_LN3	409	A
H034	409	A
H075	409	A
H157	409	A

H418	409	A
H331	4238	A
H446	46	A
42_1_R2	48	A
H002	48	A
53_39_AD	52	A
H625	5331	A
H604	540	A
H621	540	A
H317	5612	A
H242	607	A
58_3_Ti7	607	A
50_6_TCUA1	6174	A
59_1_AC3	635	A
H375	635	A
H529	635	A
H190	6489	A
H400	6490	A
H738	6491	A
H537	685	A
2H_315_15	6928	A
2H_255_4	6929	A
H492	697	A
H495	697	A
H497	746	A
H647	746	A
H442	747	A
H141	783	A
H218	88	A
H489	93	A
H593	93	A
29_3_DC10	1049	B1
H432	1079	B1
H120	1125	B1
H641	12222	B1
H759	129	B1
20_1_TC6	1304	B1
H180	1326	B1
H477	1483	B1
50_7_TCUA2	154	B1
H162	154	B1
H591	155	B1
47_3_R4	160	B1
H368	1611	B1
H039	164	B1
43_2_R17	1642	B1

33_3_R3	1727	B1
51_1_Ti1	1727	B1
H558	173	B1
73_1_Si1	2005	B1
62_4_Si7	223	B1
8_3_DC15	270	B1
43_1_R3	2773	B1
H420	2783	B1
H583	317	B1
7_14_6A	327	B1
H329	348	B1
H401	3570	B1
CD_57_LN	3759	B1
H727	3765	B1
H664	442	B1
6_14_8	453	B1
H095	453	B1
H110	453	B1
H467	453	B1
H494	453	B1
68_2_Ti3	517	B1
68_4_TC14	517	B1
H665	517	B1
24_1_Si1	58	B1
44_1_Ti4	58	B1
53_1_AC6	58	B1
H260	58	B1
H431	58	B1
H573	58	B1
H220	602	B1
50_4_ACUA1	6169	B1
37_2_AC20	6171	B1
18_1_DC10_redo	906	B1
H672+13133:144	906	B1
47_1_TC4	110	B2
12_GNB_003	1193	B2
12_GNB_025	1193	B2
12_GNB_036	1193	B2
GNB_2846	1193	B2
GNB_3401	1193	B2
48_1_R18	12	B2
62_1_Ti3	12	B2
H588	126	B2
62.19	127	B2
12_2_Ti13	127	B2
CD_52_IM4	127	B2

DMG_1993	127	B2
DMG_1998	127	B2
H744	127	B2
H183	131	B2
H757	131	B2
10_1_R6	131	B2
125.24	131	B2
32_2_R12	131	B2
33_1_TC19	131	B2
55_1_AU4	131	B2
72_2_AC9	131	B2
9_5_R1	131	B2
H090	131	B2
H186	131	B2
19_1_TC4	14	B2
H223	141	B2
H689	141	B2
H707	141	B2
45_3_DC2	144	B2
H176	144	B2
H578	1855	B2
H556	1867	B2
H630	1867	B2
H504	1871	B2
H515	1877	B2
H063	1915	B2
H146	1916	B2
H283	1917	B2
H320	1918	B2
54_1_TC4	1919	B2
H324	1919	B2
CD_1_IM3	2622	B2
58_2_AC1	28	B2
DMG_2015_G1	2800	B2
H522	3276	B2
H246	357	B2
H354	379	B2
13_1_C11	420	B2
32_1_DC18	420	B2
13_3_TC6	4230	B2
36_1_TC9	429	B2
57_3_TC14	537	B2
H100	538	B2
H022	5425	B2
52_2_Ti10	550	B2
69_1_AU1_1	569	B2

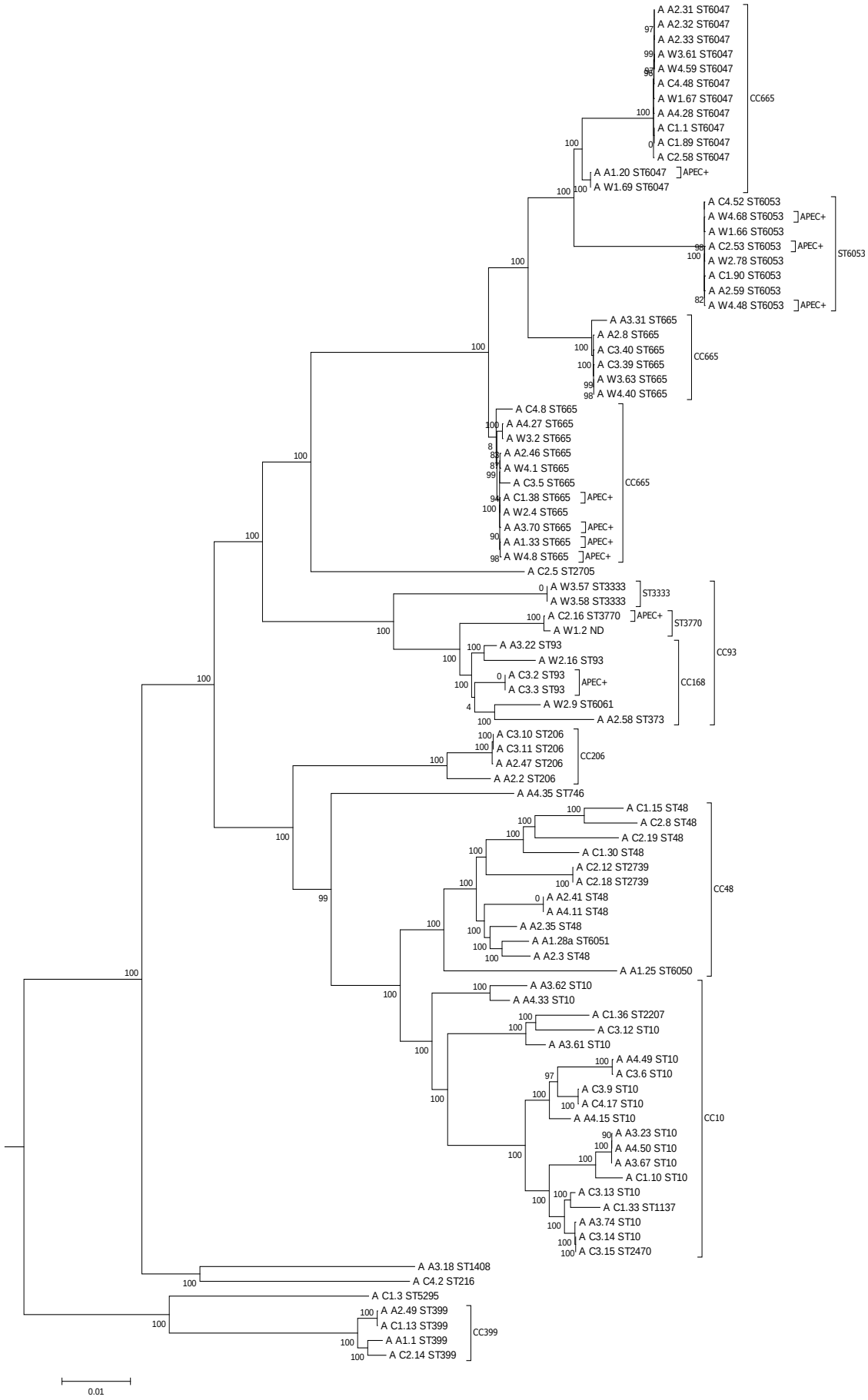
70_2_Ti12	569	B2
72_6_Ti12	569	B2
29_2_DC4	589	B2
H648	6166	B2
H217	646	B2
H001	681	B2
H438	73	B2
18_3_Ti5	73	B2
2_1_P20	73	B2
20_5_R7	73	B2
21_1_TC7	73	B2
23_1_TC4	73	B2
35_MA_AUDC	73	B2
CD_29_IM6	73	B2
H020	73	B2
H378	73	B2
H411	73	B2
H560	73	B2
60_1_Ti1	80	B2
63_2_TC7	80	B2
15_5_C3	91	B2
H021	91	B2
H333	91	B2
H468	91	B2
H655	91	B2
2H_265_13	95	B2
2H_276_5	95	B2
2H_31_1	95	B2
2H_327_14	95	B2
2H_68_14	95	B2
35_9_Ti14	95	B2
39_1_AC10	95	B2
40_1_R9	95	B2
45_1_Ti1	95	B2
46_1_Ti2	95	B2
5_1_TC12_redo	95	B2
52_1_Ti3	95	B2
56_2_AC5	95	B2
6_1_TC16	95	B2
61_1_Ti1	95	B2
61_2_AC9	95	B2
62_2_Ti6b	95	B2
70_4_DC20	95	B2
BS11	95	B2
BS117	95	B2
BS14	95	B2

BS142	95	B2
BS150	95	B2
BS157	95	B2
BS165	95	B2
BS17	95	B2
BS20	95	B2
BS36	95	B2
BS45	95	B2
BS58	95	B2
BS76	95	B2
BS84	95	B2
BS87	95	B2
CD_27_IM1	95	B2
CD_34_LN	95	B2
CD_62_LN	95	B2
DMG_2012	95	B2
H008	95	B2
H062	95	B2
H077	95	B2
H083	95	B2
H087	95	B2
H104	95	B2
H112	95	B2
H113	95	B2
H140	95	B2
H144	95	B2
H148	95	B2
H151	95	B2
H154	95	B2
H219	95	B2
H244	95	B2
H249	95	B2
H347	95	B2
H355	95	B2
H358	95	B2
H365	95	B2
H413	95	B2
H434	95	B2
H437	95	B2
H552	95	B2
M587877	95	B2
M638178	95	B2
M638488	95	B2
M638866	95	B2
M644693	95	B2
M652535	95	B2

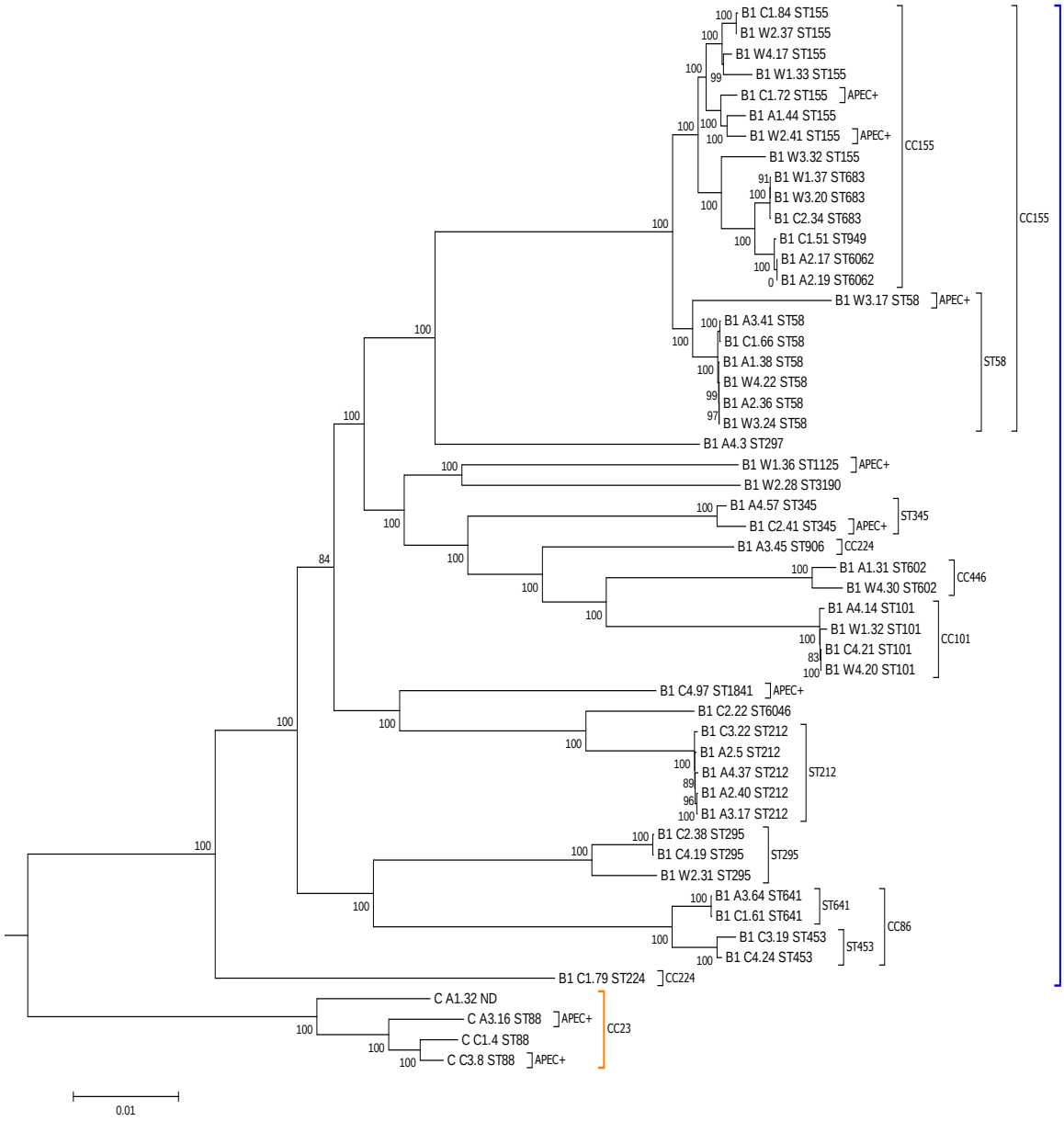
M652995	95	B2
M653497	95	B2
M658539	95	B2
M660875	95	B2
M660876	95	B2
M663105	95	B2
M663475	95	B2
M663940	95	B2
M664361	95	B2
M665752	95	B2
M666694	95	B2
M668546	95	B2
M671335	95	B2
M673438	95	B2
M675220	95	B2
M676943	95	B2
M710019	95	B2
H284	998	B2
7_14_25	1671	C
H282	23	C
2H_145_14	88	C
H055	88	C
H073	88	C
H131	88	C
H342	88	C
51_2	108	D
65_1_AC2	1177	D
25_1_ADC_2	132	D
67_3_Ti10	132	D
72_4_Ti9	132	D
47_4_R1	1567	D
H006	1881	D
14_1_R13	3056	D
8-5-Ti14	3300	D
8_1_AC8	3300	D
1_3_Ti10	349	D
7_3_R4	349	D
25_2_DC7	362	D
29_1_DC3	362	D
29_4_DC8	362	D
67_1_Ti13	362	D
68_3_TC19	362	D
70_3_Ti8	362	D
71_2_Ti5	362	D
72_5_Ti14	362	D
56_3_AC1	6170	D

64-AC5	69	D
64-TC5	69	D
11_1_Ti6	69	D
22_1_Ti17	69	D
4_1_C3	69	D
46_2_Ti11	69	D
64_AC3	69	D
70_1_AC3	69	D
41_1_Ti9	720	D
66_1_AC1	938	D
26_1_C13	973	D
6_14_6B	6173	E
H711	1771	E
71_1_AC1	1674	F
12_GNB_213	354	F
2H_40_2	354	F
GNB_2829	354	F
26_AU_GLYBX	457	F
17_1_R20	59	F
2H_273_2	59	F
2H_474_1b	59	F
53_30_AD	59	F
H071	59	F
H336	59	F
H396	59	F
H615	59	F
H719	59	F
H038	62	F
H352	62	F
H366	62	F
12_GNB_311	648	F
GNB_2711	648	F
GNB_2781	648	F
GNB_2809	648	F
GNB_2838	648	F
GNB_3697	648	F
2H_103_1	117	H299
2H_290_1	117	H299
2H_434_21	117	H299
H030	117	H299
H561	117	H299
2H_327_20	657	H299
H498	10055	
41-1-TC15	3301	
64-5-AC12	3302	
H414	3483	

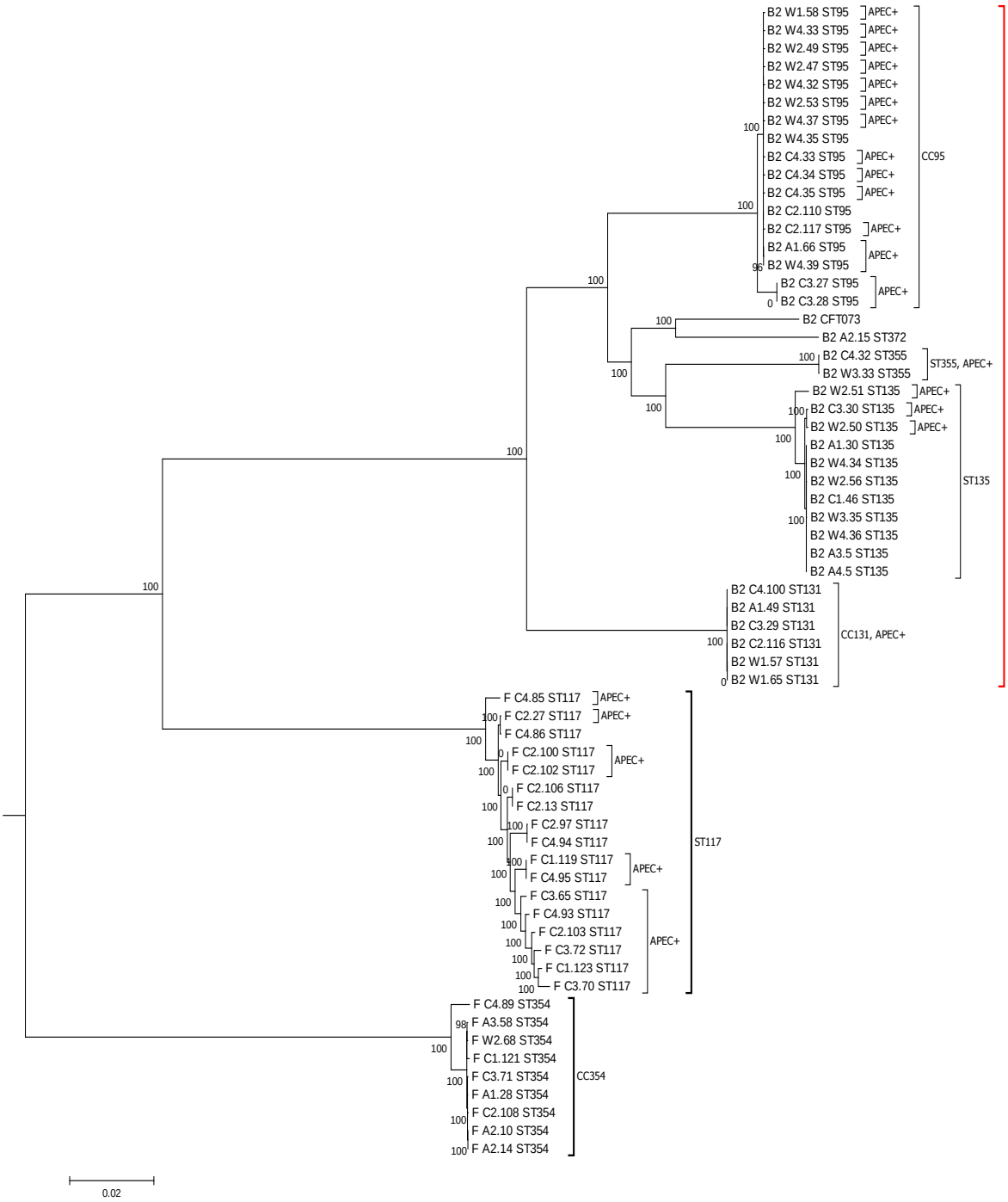
H765	3571	
30-2-R7	648	
64-1-AC1	767	
64-AC7	767	



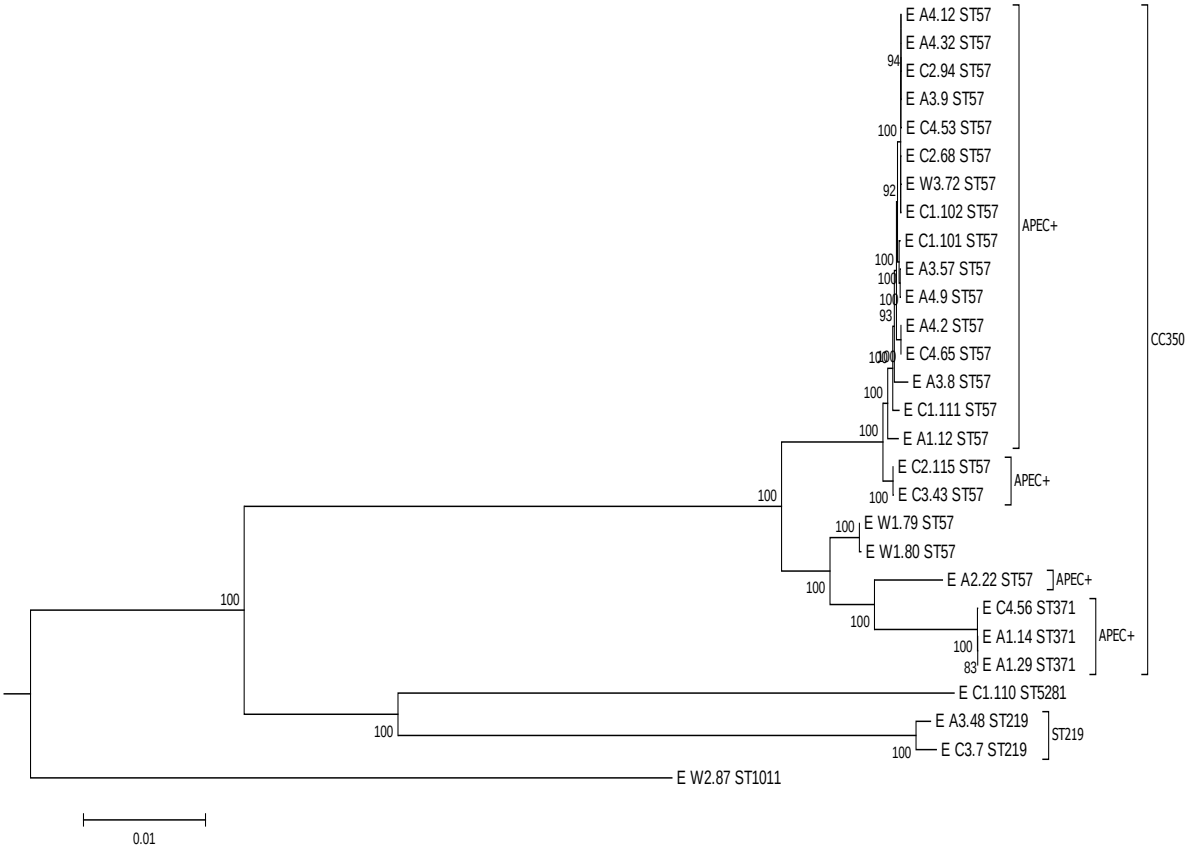
Supplemental Figure 3.1a. Phylogenetic tree (zoomed image of Fig. 3.1) representing distribution of phylogroup A isolates inferred using core genome SNPs by HARVEST.



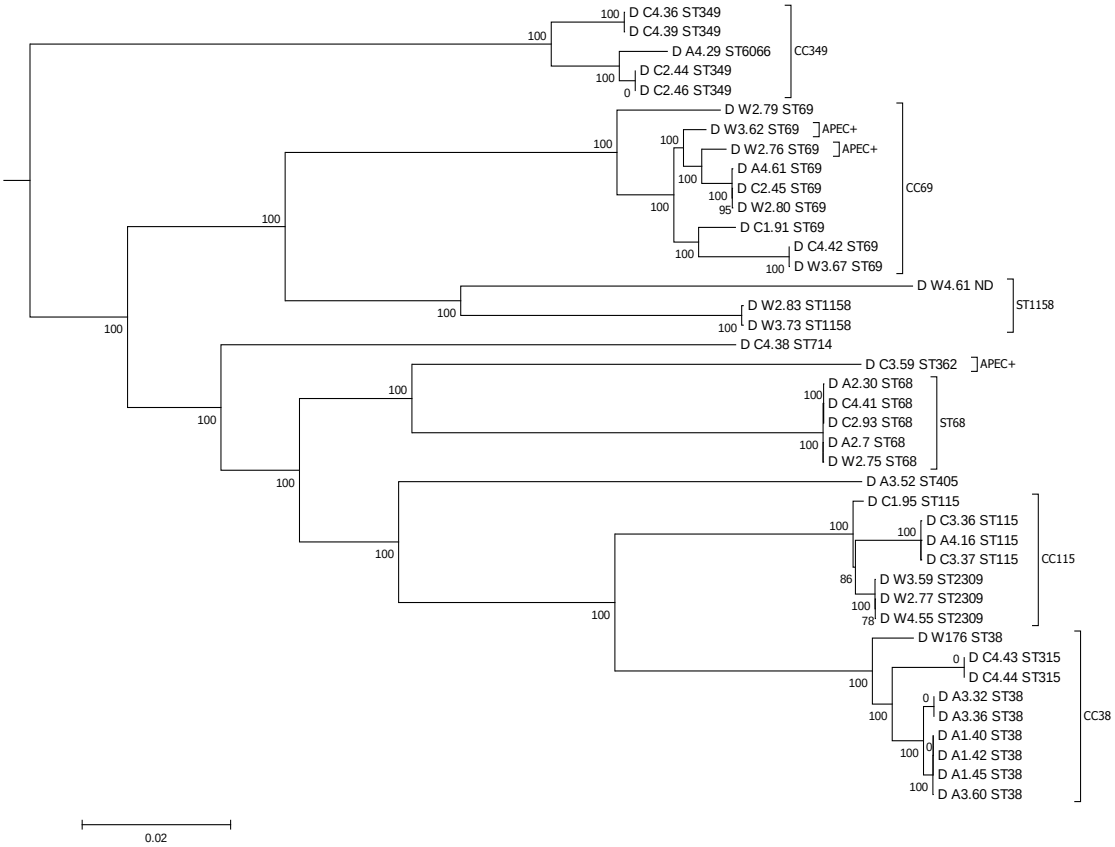
Supplemental Figure 3.1b. Phylogenetic tree (zoomed image of Fig. 3.1) representing distribution of phylogroups B1 and C isolates inferred using core genome SNPs by HARVEST.



Supplemental Figure 3.1c. Phylogenetic tree (zoomed image of Fig. 3.1) representing distribution of phylogroups B2 and F isolates inferred using core genome SNPs by HARVEST.



Supplemental Figure 3.1d. Phylogenetic tree (zoomed image of Fig. 3.1) representing distribution of phylogroup E isolates inferred using core genome SNPs by HARVEST.



Supplemental Figure 3.1e. Phylogenetic tree (zoomed image of Fig. 3.1) representing distribution of phylogroup D isolates inferred using core genome SNPs by HARVEST.

Chapter 4

Comparison of *Escherichia coli* in poultry meat and humans with focus on ST117, ST95, ST131 and ST69

4.1 Abstract

Poultry meat has been implicated as a reservoir and potential vehicle for the transmission of *Escherichia coli* (*E. coli*) lineages capable of causing infections in humans and which often exhibit antimicrobial resistance. In this study, *E. coli* sequence types (STs) 95, 131, 69 and 117 isolated from human and poultry meat samples in Canberra, Australian Capital Territory, Australia, were selected for comparison using whole genome sequencing approach. Previous studies have indicated the presence of these STs identified in human extra-intestinal pathogenic *E. coli* (ExPEC), avian pathogenic *E. coli* (APEC) and also in retail poultry meat (Manges et al., 2015, Dissanayake et al., 2014). In our study, ST131 and ST69 were detected in lesser proportions in poultry meat as compared with ST117 and ST95. Resistance was seen common to antibiotics like ampicillin (ST131), tetracycline (ST95), sulfamethoxazole-trimethoprim (ST117), but in contrast to other studies, very low level of resistance to quinolone, and no resistance to carbapenem and 3rd generation cephalosporins were observed. A number of strains harboured virulence genes commonly associated with human ExPEC and APEC like *iss, hlyA, ibeA, iutA, sitA, fimH, cva* with an average of 29.8 (ST95), 29.5 (ST131), 18.4 (ST69) and 25.1 (ST117) virulence factors out of 69 tested. The strains belonging to ST69 and ST117 had diverse serotypes, unlike ST95 (O1:H7, H7) and ST131 (O25:H4). Overall, ST131, ST95 and ST69 poultry meat and human strains clustered quite separately and were significantly different from each other. On the other hand, a number of the ST117 poultry meat and human strains analysed were significantly similar, suggesting a potential poultry meat to human transfer or vice-versa. The data from this study indicates that poultry meat ST117 and ST95 strains could serve as potential zoonotic ExPEC vehicles to humans, but with low risk transmission.

4.2 Introduction

Escherichia coli is one of the most studied, genetically variable Gram negative bacteria. The organism is associated with 75% to 90% of urinary tract infections (UTIs) and other severe human infections like neonatal meningitis, bloodstream infections (septicemia or bacteremia) and sepsis, which are all associated with extra-intestinal pathogenic *E. coli* (ExPEC). Colibacillosis is caused by avian pathogenic *E. coli* (APEC) and is a significant

problem in the poultry industry (Ewers et al., 2007). APEC has also been suggested to be capable of causing diseases in humans (Johnson et al., 2007). Virulence factors (VF) like *eae, eilA, iss, hlyA, ibeA, iutA, sitA, fimH, usp,* and bacteriocins like *cvi/cva*, often coupled with antimicrobial resistance determinants, play a major role in the intestinal and extra-intestinal pathogenesis of *E. coli* (Sheikh et al., 2014, Johnson and Russo, 2005).

Poultry meat, more so than beef or pork, has been found to be potential reservoirs of ExPEC strains that often also exhibit multi-drug resistance (Bergeron et al., 2012, Manges and Johnson, 2012). Chicken meat is now the most consumed meat in Australia, with an average annual consumption of 46.2 kilograms per person, and consumption is continuing to increase (ABARES, 2016). The popularity of the meat is largely due to its affordability compared to other meats like beef and pork, and partly because of its nutritional value, as it serves as a good source of low-fat meat protein (Charlton et al., 2008).

The *E. coli* strains associated with ExPEC in humans widely belong to the sequence types ST73, ST95, ST131 and ST69 (Mora et al., 2013, Johnson et al., 2012, Manges and Johnson, 2013). ST73, ST95 and ST131 belong to phylogroup B2, while ST69 is a member of phylogroup D (also termed as clonal group A). On the other hand, ST117 is a known poultry lineage ST, commonly isolated from poultry meat as well as humans (Manges, 2016, Bergeron et al., 2012). Not much is known about the role of ST117 as a potential ExPEC in humans, but it has been identified as a potential avian pathogen (Dissanayake et al., 2014, Maluta et al., 2014).

A number of molecular epidemiology studies (using traditional DNA-based typing methods) focusing on *E. coli* strains belonging to the lineages ST117, ST95, ST131 and ST69 from humans and poultry meat (Ghodousi et al., 2016, Manges et al., 2015, Vincent et al., 2010) have indicated that poultry and poultry meat products are a possible reservoir of *E. coli* capable of causing extra-intestinal infection in humans. Epidemiological studies conducted in specific human populations noted that highly similar strains were isolated from geographically matched human infection samples and

poultry meat (Jakobsen et al., 2012, Manges et al., 2007, Johnson et al., 2005a, b). These studies used traditional molecular typing methods like pulsed-field gel electrophoresis (PFGE) (Jakobsen et al., 2012, Manges et al., 2007) and random amplified polymorphic DNA (RAPD) (Johnson et al., 2005a) to determine strain similarities. Evidence from these studies thus suggest that the food we consume contributes, to some level, in transferring ExPEC-related isolates by cross-contamination, and also that food-producing animals serve as ExPEC reservoir (Manges, 2016, Mitchell et al., 2015, Clermont et al., 2011, Leverstein van-Hall et al., 2011, Jakobsen et al., 2010, Ramchandani et al., 2005, Johnson et al., 2003). A limitation of these studies is that they have been based on similarities in their ST membership and or similarities in their VF and/ or resistance profiles (Singer, 2015). A whole genome sequencing (WGS)-based study by de Been et al. (2014) made observations quite different from the molecular-based epidemiological studies. Even though some strains isolated in poultry meat were of the same ST as the human ExPEC strains (like STs 95, 131, 69), shared serotypes, harboured similar VFs, and sometimes resistance genes as well, most often they were not identical and clustered separately from human isolates. On the other hand, only a few studies have focused on WGS-based approach (de Been et al., 2014, McNally et al., 2016, Hansen et al., 2016, Mo et al., 2016, Hasman et al., 2015). A study by McNally et al. (2016) presented WGS-based data supporting the possibility of ST131 lineage as a potential zoonotic pathogen. An extensive review on foodborne ExPEC by Singer (2015) rightly suggested that studies with better experimental design and preferably WGS approach is required for transmission route, source tracing and comparative studies.

To the best of our knowledge, no extensive study has been carried out on poultry meat (by consumption, handling or processing) as a potential reservoir and vehicle of transmission of infection inducing *E. coli* with zoonotic potential in Australia. In this study, we compared the whole genome data of *E. coli* strains belonging to ST117, ST69, ST95 and ST131 collected from human clinical samples (H) and poultry meat (PM) samples, during 2013 to 2015 in Canberra, ACT, Australia.

4.3 Materials and methods

4.3.1 *Sampling*

Retail poultry meat products (n = 306) were sampled during the summer of 2013 (November to December), then autumn (April) and winter (August) of 2014, from sixteen shops representing three major supermarket chains and independent butcheries located in four of the main town centres in Canberra, Australian Capital Territory (ACT), Australia. Breast fillet, mince, thigh, wings and whole meat from conventional sources were collected. In addition, free-range and organic breast fillets were also sampled. Each sample unit consisted of at least 100 g of packaged meat portions. The meat samples were stored and transported in cooler boxes with packaged ice blocks for providing refrigerated conditions, similar or as close to the conditions presented to the consumer at the time of purchase from where they have been collected/ purchased.

The human clinical *E. coli* samples (n = 389) were isolated between 2014 and 2016 from urine, blood and faeces by the Microbiology Unit of the Canberra Hospital, ACT, Australia.

4.3.2 *Isolation of E. coli*

The 306 poultry meat samples were processed within an hour from each of their sampling time. A small portion of the meat (20 g each) were transferred to sterile stomacher bags and homogenized using a stomacher for 3 minutes, with 180 mL of Maximum Recovery Diluent enrichment broth (pre-enrichment) with a final 1:10 dilution. The stomacher bags have a filter membrane which separates the meat pieces with the homogenate solution. The diluted homogenate (filtrate) were then recovered using sterile syringes, plated onto MacConkey agar plates (Acumedia, Neogen) for isolation of *E. coli* using spread plate method.

Also 1 mL each of diluted sample homogenate from the bags were used to inoculate flasks containing 9 mL of Lauryl Sulfate broth (Acumedia, Neogen) and 9 mL of Luria-Bertani (LB) broth supplemented with vancomycin (20 mg/L) for further enrichment. After 18 hours of shaking incubation at 35^{o}C, the enriched broth cultures were directly

dilution streaked onto MacConkey agar plates. The enriched broth cultures (100µL each) were also lawn plated onto MacConkey agar plates with eight different antimicrobials (amoxicillin-clavulanic acid, nalidixic acid, nitrofurantoin, gentamicin, ampicillin, trimethoprim-sulfamethoxazole, ceftiofur and tetracycline) for antibiotic selection of *E. coli* cultures. The suspected *E. coli* cells (lactose positive) which grew within the zone of inhibition of the antibiotic discs were then dilution streaked onto MacConkey agar plates for isolation of single *E. coli* colonies. The clinical human samples were directly dilution streaked onto MacConkey plates and single colonies were picked for further characterization.

4.3.3 *Strain characterization*

The retail poultry meat (PM) and clinical human (H) strains selected for this study were from two extensive sampling carried out during 2013 to 2015 in Canberra, ACT, Australia. Prior to selection for whole genome sequencing, the *E. coli* isolates (n = 3415) were characterized for their phylogroup membership using Clermont et al. (2013). Isolates from the same meat samples were then REP-PCR (Repetitive Element Palindromic PCR) fingerprinted using ERIC (Versalovic et al., 1991) and CGG (Adamus-Bialeket al., 2009) primers. One example of every REP-fingerprint type from each meat sample was maintained for further characterization. All REP-types belonging to phylogroup B2 were selected for whole genome sequencing. A subsample (n = 9) of strains identified as phylgroup F were selected for WGS. The detailed protocols for the isolation, molecular characterization of *E. coli* and phenotypic antimicrobial susceptibility test were as per Blyton et al. (2015). A total of 619 isolates from humans were available and these isolates were characterized for their phylogroup membership and then isolates belonging to CC95, CC131 and CC69 were identified using the B2 subtyping (Clermont et al., 2014) and Doumith method (Doumith et al., 2015). A significant fraction of the strains belonging to STs 95, 131 and 69 had been whole genome sequenced as part of other studies. ST117 isolates from humans had been identified in the course of a WGS study of phylogroup F strains in Australia (Vangchhia et al., 2016). The metadata of the strains is given in Supplemental Table 4.1 and 4.2.

4.3.4 *Whole genome sequencing*

Genomic DNA for whole genome sequencing was isolated using isolate II Genomic DNA kit extraction (Bioline). DNA was quantified using Qubit dsDNA (double stranded DNA) BR (broad range) Assay kit (Invitrogen). DNA (0.5 ng) at 0.2 ng/µL was used for preparing the sequencing libraries using the Nextera XT DNA Sample Preparation kit (Illumina) and the Nextera XT index kit (Illumina). Whole genome DNA sequencing was performed on Illumina MiSeq platform using a 600 cycle MiSeq Nextera XT version3 sequencing chemistry. The raw genomic sequencing data files generated from Illumina's MiSeq platform were then assembled as de novo genome sequences and exported as FASTA files using CLC Genomics Workbench V9.0.

4.3.5 *In silico MLST and Serotyping*

The *E. coli* strains were assigned to sequence types using the University of Warwick MLST scheme (Wirth et al., 2006) (www.mlst.warwick.ac.uk). The O and H serotype of the isolates was determined in silico using the web-based tool SeroTypeFinder (Joensen et al., 2015) tool available on the Centre for Genomic Epidemiology (CGE) website (www.genomicepidemiology.org). The virulence gene profile of the strains was determined using the VirulenceFinder tool (Joensen et al., 2014), while the BLAST feature of CLC Genomics Workbench V9.0 was used to screen for ExPEC virulence factors not determined by the CGE website. The ResFinder tool (Zankari et al., 2013), Centre for Genomic Epidemiology (CGE), was used to identify the plasmid-associated antimicrobial resistance determinants, while the Resistance Gene Identifier (RGI) tool available on The Comprehensive Antibiotic Resistance Database website (http://arpcard.mcmaster.ca/) was used to find both chromosomal and plasmid resistance genes determinants (McArthur et al., 2013). A strain was classified as multi-drug resistant (MDR) when two or more antimicrobial class resistance genes were detected.

4.3.6 *Phylogenetic tree and variable gene content inference*

HARVEST suite (Treangen et al., 2014) was used for inferring phylogenetic trees using core genome single-nucleotide polymorphisms (SNPs) for constructing the tree.

Recombination detection option was used in Harvest. Specific reference strains were used in the HARVEST alignments: UTI89 for ST95, EC958 for ST131 and UMN026 for ST69. There is no reference strain available for ST117.

The program MAUVE (Darling et al., 2010) was used for genomic alignment. Draft assemblies were first reordered using MAUVE and the appropriate reference strains. ST117 strains were reordered using S88 strain. MAUVE was used to align the strains belonging to each ST. The MAUVE backbone file was used to determine the variable genome regions present among the strains belonging to each ST. DNA blocks present in all strains or just one strain were excluded and the remaining blocks were scored as either present or absent using Excel spreadsheet. The DNA blocks were further analyzed for their gene identity using an annotated genome as reference and genes that were absent in the reference genome were investigated using BLASTn (nucleotide Basic Local Alignment Search Tool) (Altschul et al., 1990).

4.3.7 *Plasmid content*

The gene content of the plasmids in ST117 and ST95 strains was inferred using an iterative process. First, the contigs containing antimicrobial resistance genes were identified, and contigs with incompatibility (Inc) group (or plasmid replicon type) matches were identified using PlasmidFinder (Caratolli et al., 2014) tool also available in the CGE website. Contigs containing other known plasmid-borne genes (examples are colicin B and *iroN*) were also identified. The plasmid-associated contigs were then joined into a 'plasmid assembly' for each isolate and the plasmid assemblies were aligned with the full assemblies of all isolates belonging to the same ST. Additional plasmid-associated contigs were identified, and the process was repeated until no new plasmid-associated contigs could be discovered. The resulting inferred plasmid assemblies were compared against the National Center for Biotechnology Information (NCBI) database by using BLAST to confirm that the resulting plasmid assemblies did not contain genes normally associated with the chromosome. The plasmid assemblies (contigs) for each strain were then aligned using MAUVE and the variable gene content of each strain was extracted from the backbone file.

4.4 Results

4.4.1 *ST117*

ST117 strains yield a phylogroup F genotype using the Clermont et al. (2013) method. Of the phylogroup F isolates from poultry, 26 were sequenced and 65.4% were actually ST117, the balance represented ciprofloxin resistant F strains belonging to ST354. In total there were 6 ST117 strains from humans and 17 isolates from poultry available for comparison.

A total of 16 serotypes were present out of 23 ST117 strains. Among the 17 poultry meat (PM) ST117 strains, 10 different serotypes were identified, namely O45:H4 (n = 3), O45:H18 (n = 2), O53:H4 (n = 2), O71:H4 (n = 2), O111:H4 (n = 2), H4 (n = 2), O119:H4 (n = 1), O143:H4 (n = 1), O149:H10 (n = 1) and O161:H10 (n = 1). None of the 6 human (H) isolates shared the same serotype. The serotypes present in H strains were O143:H4, H4, O111:H4, O114:H4, O7:H45, O161:H4 and O24:H4. The shared serotypes present in both humans and poultry meat isolates were O111:H4 and O143:H4. These serotypes have been consistently observed in different studies (Mora et al., 2012, Vincent et al., 2010).

Fourteen poultry meat and 5 human ST117 strains possessed resistance genes (82.6%), a chromosomal-mediated quinolone resistance mutation in *gyrA* was found in 2 ST117 poultry meat strains; all other resistance determinants were plasmid-mediated. 2 poultry meat strains (C2-100 and C2-102) and 1 human strain (434-21) harboured same resistance genes namely *strA, strB, sul1, sul2, tetB, dfrA5*. Out of the poultry meat strains, 4 strains (C2-13, C2-103, C2-106 and C4-93) had *sul2*, 2 (C2-97, C4-94) had *blaCMY*-2, C1-119 had *blaTEM-1B, dfrA5* and *sul1*, C2-27 had *tetA* and *tetC*, C4-95 had *blaTEM-1B* and *dfrA5*, C4-85 had *tetA* and *dfrA5,* C3-70 had *strA, strB, sul2* and *catA1,* and C3-72 had *blaTEM-1B* resistance determinants. Both C3-70 and C3-72 had *gyrA* quinolone resistance mutations. For human strains, H030 possessed resistance genes *blaTEM-1B, dfrA17, sul1, tetA, aadA5* and *oqxB,* 103-1 had *blaTEM-1B, strA, strB*, *sul2, tetA* and *dfrA5,* 290-1 had *blaTEM-1B* and *tetB,* and H299 had *blaTEM-1B, tetA, tetC,*

sul2, strA, strB and *dfrA5.* 7 poultry meat (41.2%) and 5 human (83.3%) strains were classified as multi-drug resistant with >2 antimicrobial classes resistance determinants.

With respect to virulence factors in ST117 strains, the virulence factors harboured by all the strains were *vat, eaeH, chuA, iss, lpfA, prfB, etsC, fimA, fimH, iutA, ompT* (both chromosomal and plasmid), *iucC* and *sitA* (except in H299), or were common: *ireA* (91%), *iroN* (87%), *traT* (83%), and *ybtS* (74%) (Supplemental Table 4.2). All human strains analysed and 9 out of 17 poultry meat strains had two copies of *iss* (one plasmid and one chromosomal), while H299 had three copies. Bacteriocins were common; microcin H47 (*mchE*) was present in all 6 human (100%) and 10 poultry meat strains (69%). The colicins and microcins harboured are as represented in (Fig. 4.1a). All the strains analysed in this study harboured incompatibility group FIB (IncFIB) and 91.3% had IncFII additionally (Fig. 4.1c).

Principal coordinates analysis (PCA) revealed that the ST117 strains fell into three groups (Fig. 4.1b). One cluster consisted of only poultry meat isolates, another consisted of mostly poultry meat isolates together with 1 human isolates. The final cluster consisted of mostly human isolates but also included 2 poultry meat isolates. PERMANOVA analysis of the Jaccard distance matrix showed that, on average, poultry meat and human ST117 isolates were significantly different from each other ($p = 0.05$).

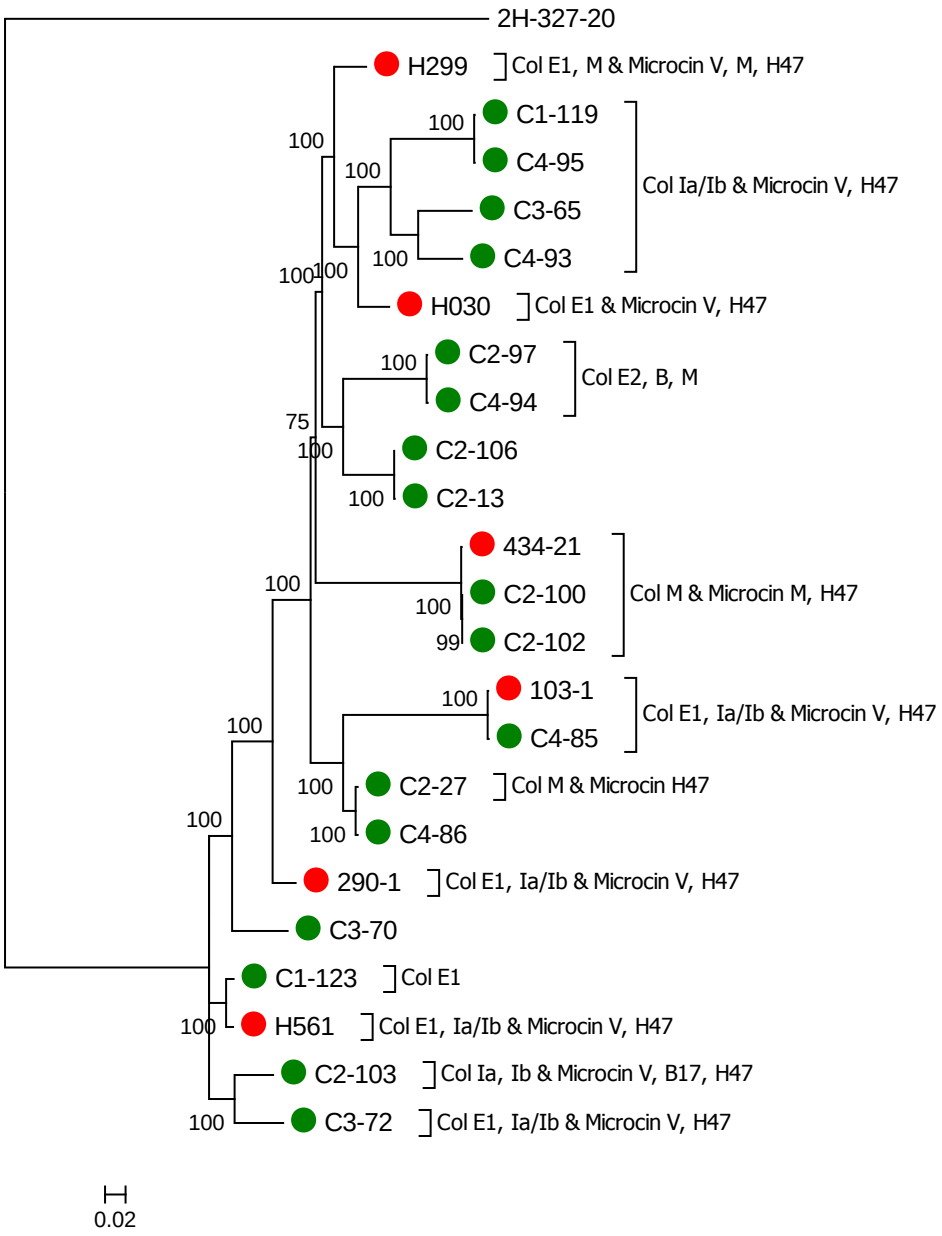


Figure 4.1a. Phylogenetic tree of ST117 strains (H=6, PM=17) using core genome SNPs inferred by HARVEST tools. Green dots = poultry meat (PM) strains, red dots = human (H) strains. The H strain (434-21) clusters with two PM strains (C2-100, C2-102), shared identical serotype, virulence and resistance determinants. Bacteriocin contents are as labelled. 2H-327-20 used as a reference outgroup strain.

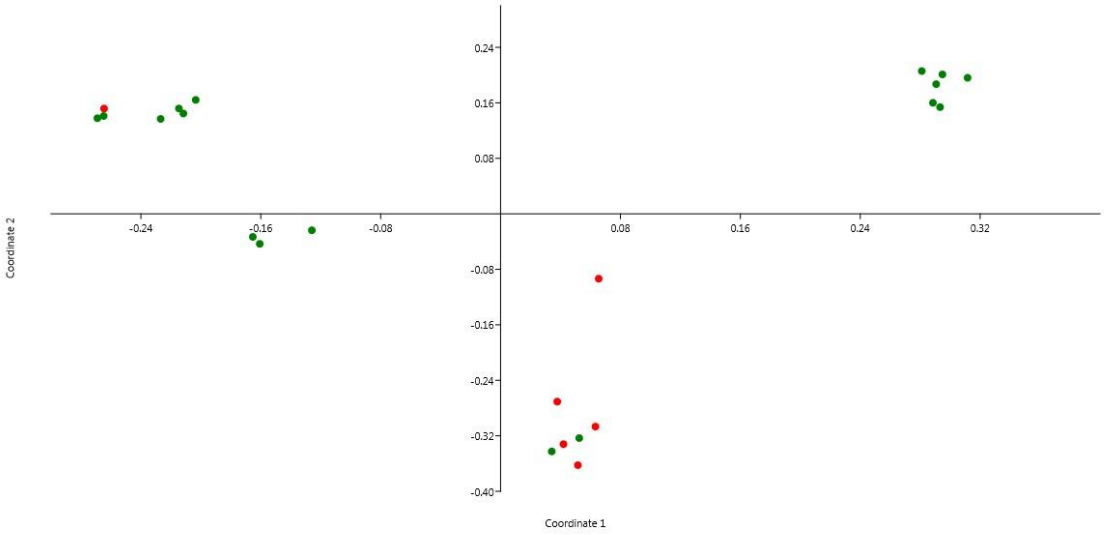


Figure 4.1b. ST117 human and poultry meat *E. coli* strains variable gene content comparison using Jaccard Principal Coordinates Analysis, obtained from MAUVE output, green dots = poultry meat strains, red dots = human strains. One H strain (434-21) is seen overlapping with two PM strains (C2-100, C2-102), sharing identical serotype, virulence and resistance determinants. Two PM strains cluster closely with five H strains.

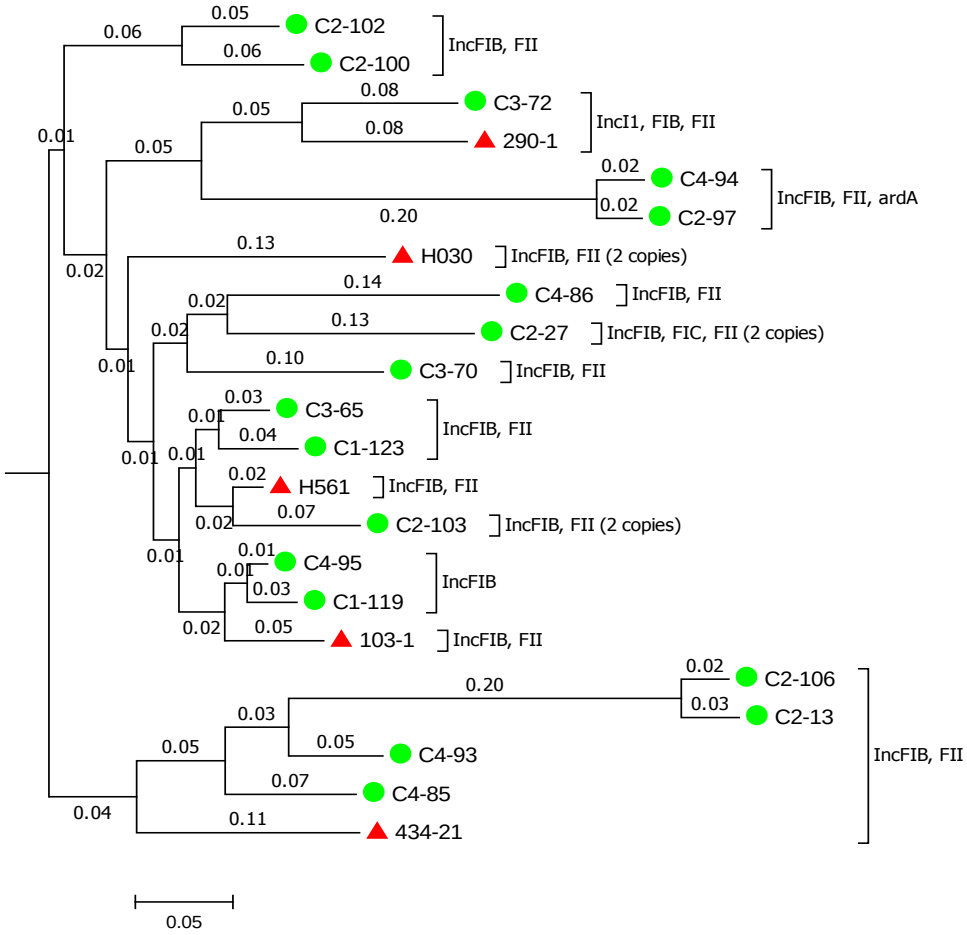


Figure 4.1c. Plasmid content inferred using MAUVE guide tree output and MEGA 6.06 for Newick neighbor-joining tree for ST117 human (red triangles) and poultry meat (green dots) strains. Identical incompatibility groups are shared between H and PM strains.

4.4.2 *ST95*

Clonal complex 95 exhibits considerable substructure (Walk and Feng, 2011). Virtually all of the poultry meat ST95 isolates belonged to a single subgroup consisting largely of strains with an O1:H7 serotype and having a *fimH27* allele. Only ST95 isolates from humans that belonged to this subgroup were included in the comparison and consequently the comparison between ST95 isolates from humans and poultry meat consisted of 12 isolates from humans and 17 isolates from poultry meat. This subgroup accounted for 70.6% of ST95 from poultry meat, but only 18% of ST95 isolates from humans.

Nine out of 17 ST95 poultry meat strains were of O1:H7 serotype and the remaining 8 were classified as H7, while 3 out of 12 human strains were H7 and the remaining 9 were O1:H7. All ST95 (PM = 17, H = 12) but 2 poultry meat and 1 human strain (89.6%) exhibited resistance to tetracycline (*tetB*). Additionally, M652995 had *blaTEM-1C* and *tetA* genes and M710019 had *blaTEM-1C, sul2, strA* and *strB* (both H strains), thus showing low levels of resistance with only 2 human strains multi-drug resistant.

ST95 strains isolated from both poultry meat (n = 17) and human sources (n = 12) also harboured high number of virulence factors namely *fimH, fyuA, ireA, kpsE, ompT (chromosomal),cah-Ag43, papG, papC, eaeH, ybtS, gad, usp* were present in all strains*, iss, traT, vat, iutA, iucC, etsC, ompT (plasmid), sitA* (96% each) and *iroN, neuC, upaG, arcA* (93%) (Supplemental Table 4.2). Microcin H47 (*mchE*), microcin V (*cva*) and colicin Ia (*cia*) were present in all but 1 human strain (M644693). Colicin E1 was present in all strains while only 2 human strains had colicin Ib. None of the strains had colicin B, M, E2 and microcin B17 (Fig. 4.2a). Also, out of the 24 strains analysed (PM = 12, H = 12), 23 strains had IncFII except 1 human strain (Fig. 4.2c).

A core gene phylogeny revealed that, on average, ST95 isolates from humans clustered separately from poultry meat ST95 isolates. Principal Coordinates Analysis (PCA) of the variable gene content of these strains revealed two distinct clusters of strains (Fig. 4.2b).

One cluster consisting largely of poultry meat isolates and the other of human isolates. PERMANOVA analysis showed that, on average, ST95 isolates from poultry meat were distinct from human ST95 isolates ($p < 0.03$).

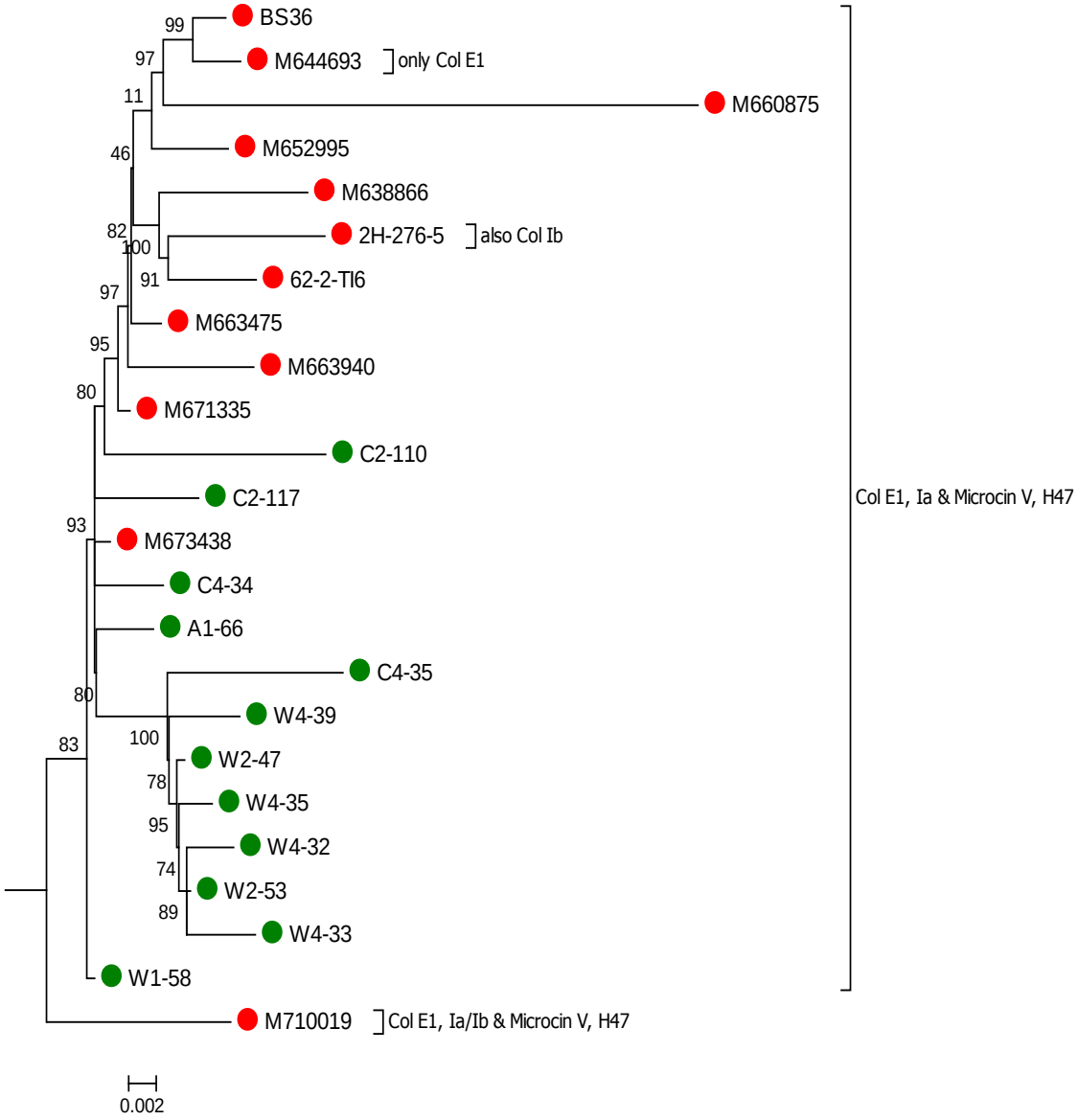


Figure 4.2a. Phylogenetic tree of ST95 strains (H=12, PM=12) using core genome SNPs inferred by HARVEST tools. Green dots = poultry meat strains, red dots = human strains. Bacteriocin contents are as labelled showing identical contents in nine H and twelve PM strains.

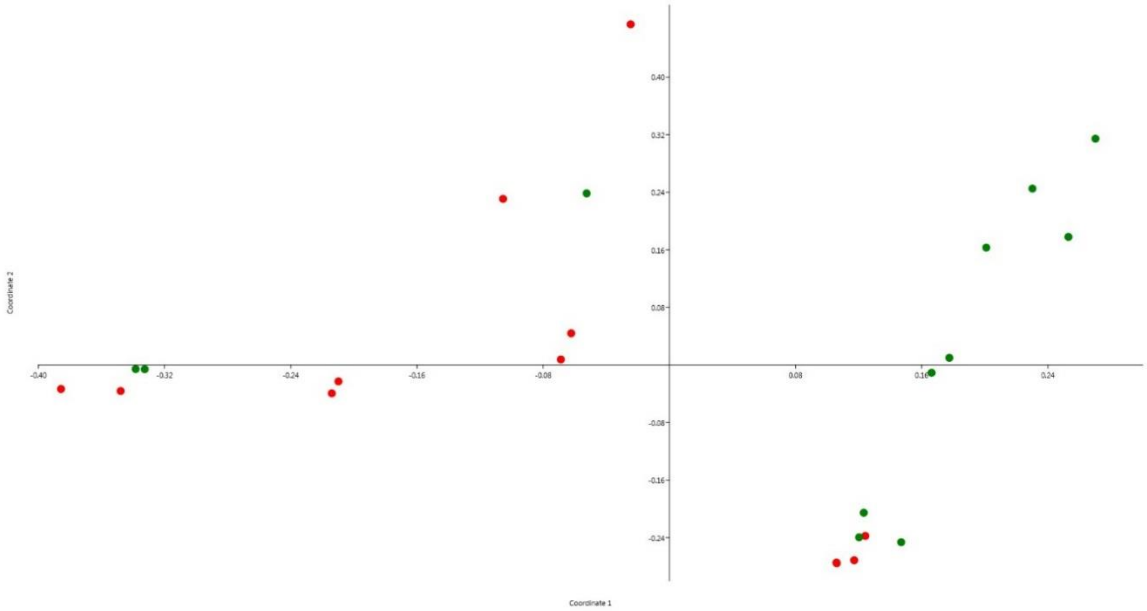


Figure 4.2b. ST95 human and poultry meat *E. coli* strains variable gene content comparison using Jaccard Principal Coordinates Analysis, obtained from MAUVE output, green dots = poultry meat strains, red dots = human strains. Two PM strains cluster with predominantly H-associated cluster. In contrast, four H strains cluster with more PM-associated distribution.

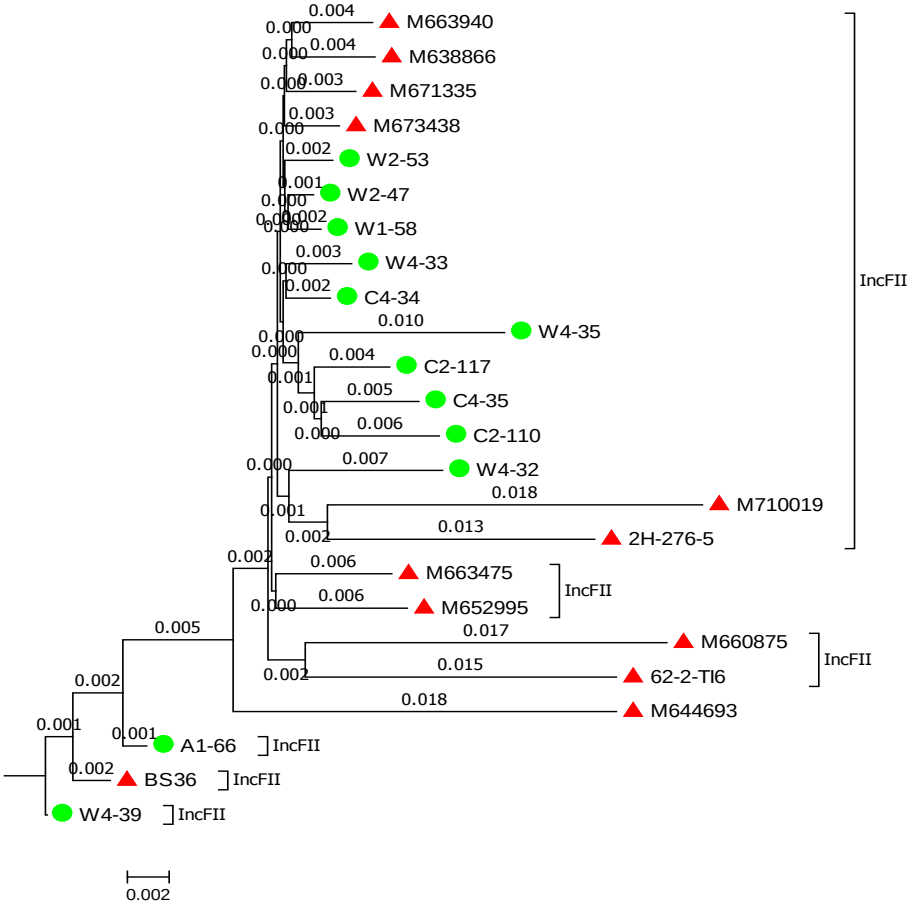


Figure 4.2c. Plasmid content inferred using MAUVE guide tree output and MEGA 6.06 for Newick neighbor-joining tree for ST95 human (red triangles) and poultry meat (green dots) strains. Incompatibility group IncFII and *tetB* plasmid-mediated resistance gene determinant were present in all strains but one H strain (M644693).

4.4.3 *ST131*

Clonal complex ST131 largely consists of three groups of strains. There are strains with an O16:H5 *fimH41* allele, strains with an O25b:H4 serotype and *fimH22* allele, and those that are O25b:H4 *fimH30*. Only 6 ST131 isolates were detected in the poultry meat samples and all were O25b:H4 *fimH22*. Consequently the poultry meat ST131 isolates were compared to O25b:H4 *fimH22* isolates from humans and there were 6 of these isolates available.

All 6 ST131 poultry meat strains exhibited ampicillin resistance (*blaTEM-1B)* and 1 H strain (M710059) had *blaTEM-1C*, while another human strain (M653835) had *blaTEM-1B,* with *tetA, strB, catA1, dfrA7* and *sul1* resistance genes. C2-116 harboured *tetA, sul1, dfrA12* and *aadA,* and C3-29 had *tetA* and *dfrA12* resistance genes additionally (both PM strains), indicating that 2 poultry meat and 1 human strain were multi-drug resistant (37.5%).

All ST131 strains had *ibeA* invasin gene and in addition, *etsC, fimH, fyuA, hra, iroN, iucC, iutA, kpsE, ompT (chromosomal and plasmid), sitA, traT, usp, cah-Ag43, ybtS, eaeH, upaG, tsh, arcA, iss, gad* were the virulence factors present in all 6 poultry meat strains and *neuC* was found in all but 1 poultry meat strain (Supplemental Table 4.2). *fimH, fyuA, kpsE, ompT (chromosomal), usp, ybtS, eaeH, arcA, gad* were all present in human strains, and *iha, sat* were present in 3 human strains while absent in all poultry meat strains. All poultry meat strains and 1 human strain had colicin Ia (*cia*), microcin V (*cva*) and H47 (*mchE*). Colicin E1/ E2, colicin B/ M were absent in all poultry meat strains while 1 human strain harboured colicin E1, along with microcin V and H47 (Fig. 4.3a). ST131 strains harboured IncFIB, FIC, FII and IncI1 plasmid MLST alleles consistently (metadata Table 4.1).

Inspection of the core genome phylogeny showed that the poultry meat ST131 isolates were distinct from the ST131 isolates from humans (Fig. 4.3b). The variable gene content

of isolates from poultry meat and humans was also distinct (Fig. 4.3b), PERMANOVA ($p < 0.003$).

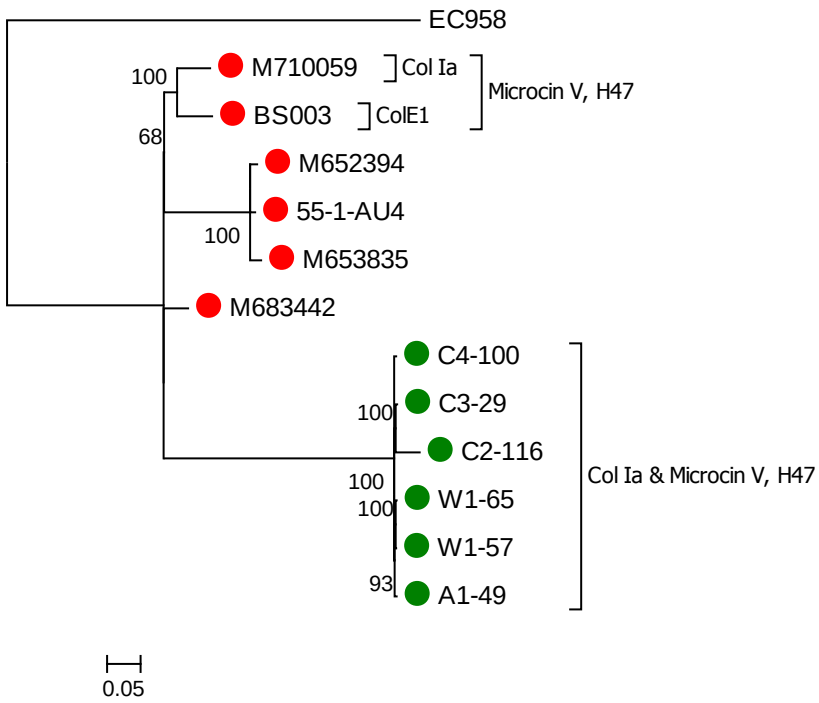


Figure 4.3a. Phylogenetic tree of ST131 strains (H=6, PM=6) using core genome SNPs inferred by HARVEST tools. Green dots = poultry meat strains, red dots = human strains. Bacteriocin contents are similar in all PM strains but present in only two H strains. EC958 used as a reference outgroup strain.

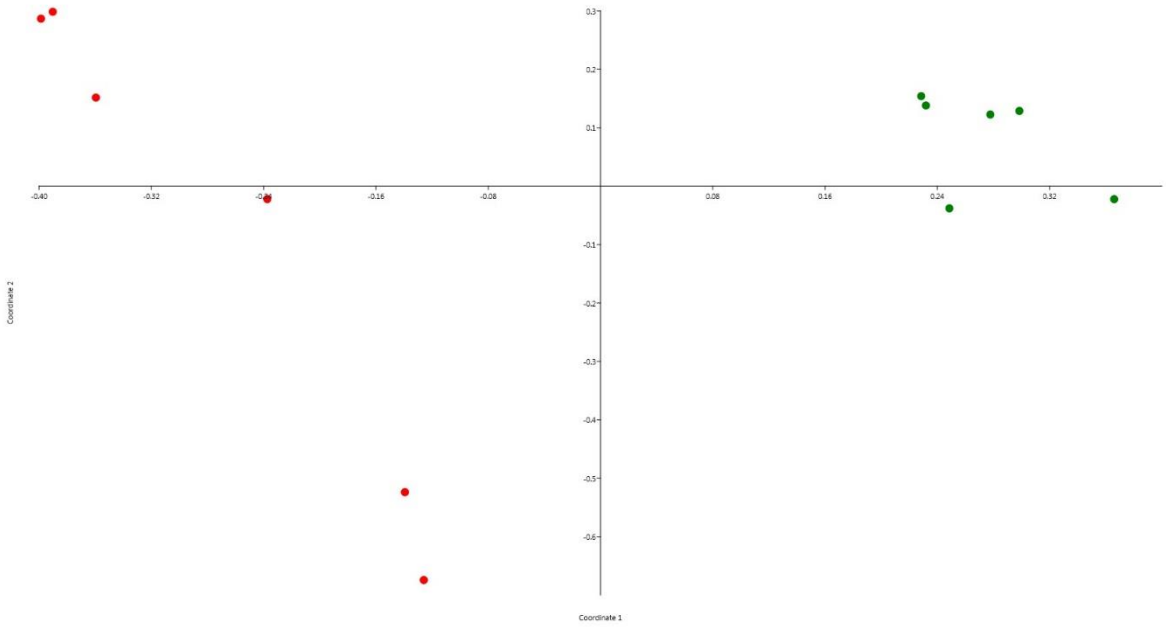


Figure 4.3b. ST131 human and poultry meat *E. coli* strains variable gene content comparison using Jaccard Principal Coordinates Analysis, obtained from MAUVE output, green dots = poultry meat strains, red dots = human strains. PM and H strains cluster distinctly separate.

4.4.4 *ST69*

There was WGS data available for 9 ST69 isolates from poultry meat, but there was WGS data for only 3 ST69 isolates from humans. The 12 ST69 isolates belonged to 4 different serotypes, namely O17/O44:H18 (n = 4; H = 1, PM = 3), O17/O77:H18 (n = 4; H = 2, PM = 2), O21:H18 (PM = 1) and H49 (PM = 3). O17/O77:H18 and O17/O44:H18 were the 2 shared serotypes between poultry meat and human strains. Only 2 poultry meat strains had plasmid-borne resistance genes, namely, *sul2, strA* and *strB* (C1-91 of serotype O17/O44:H18) and *tetC* (W2-76 of O17/O77:H18 serotype). In contrast, no human ST69 strain in this study had resistance determinants, thus showing that only 1 poultry meat strain was multi-drug resistant.

All ST69 strains were *chuA, fimH, sitA, cah-Ag43, eaeH, gad* and *lpfA_B1* positive; *iha* was present in all poultry meat strains but absent in 2 human strains. *iucC, iutA* and *kpsE* were overrepresented i.e. absent in only 1 poultry meat strain and 2 human strains and *upaG* was present in all but 1 poultry meat and absent in all 3 human strains (Supplemental Table 4.2). Also, all the strains but 1 poultry meat strain (W3-62) had *eilA* invasin gene and additionally 9 strains had *air* gene. None of the human strains analysed had colicin or microcin gene determinants. Colicin E1 was present in 7 out of 9 poultry meat strains and 2 strains additionally had colicin E2. Colicin B/ M were present in 4 strains, all strains that had colicin B/ M also possessed colicin E1 but not vice-versa. None of the strains analysed had colicin Ia/ Ib nor microcin V (Fig. 4.4a). Also, out of the ST69 strains, 2 poultry meat and 1 human strain had IncFIB alleles, and three strains with IncFII alleles, 1 poultry meat (W2-76) and 1 human (70-1-AC3) strains, in addition to IncFIB and FII, also harboured IncFIC allele (Supplemental Table 4.1).

Both the core genome phylogeny and variable gene content analyses showed the clear distinction between ST69 poultry meat isolates from the ST69 human isolates (Fig. 4.4a, 4.4b) PERMANOVA ($p < 0.03$). This points out the difference of the isolates belonging to this lineage from humans and poultry meat samples with no significant overlap between the two sources.

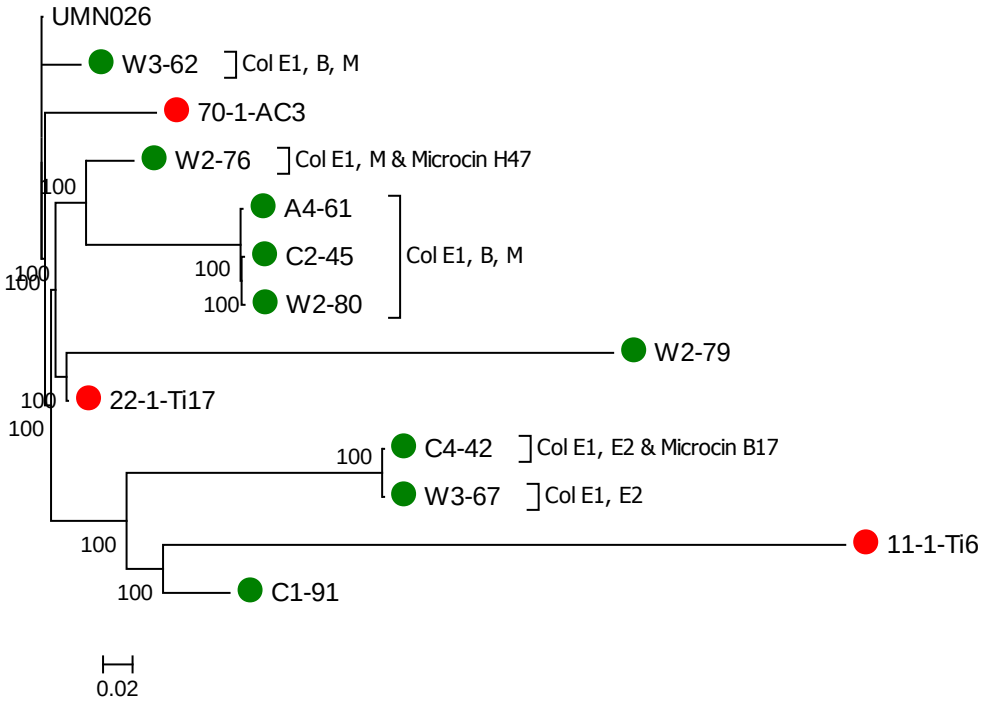


Figure 4.4a. Phylogenetic tree of ST69 strains (H=3, PM=9) using core genome SNPs inferred by HARVEST tools. Green dots = poultry meat strains, red dots = human strains. No H strain has any bacteriocin content. UMN026 used as a reference outgroup strain.

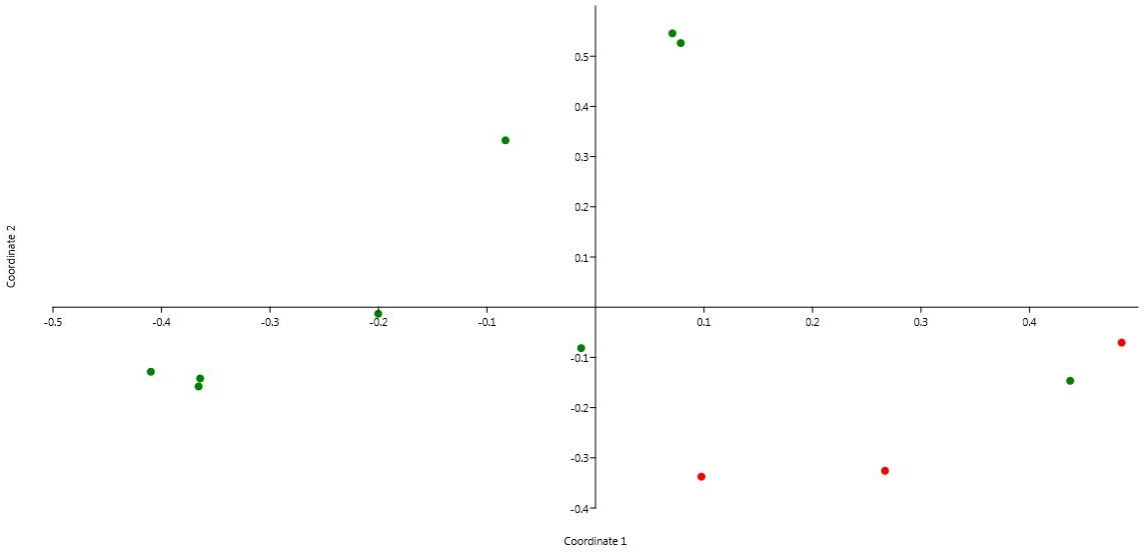


Figure 4.4b. ST69 human and poultry meat *E. coli* strains variable gene content comparison using Jaccard Principal Coordinates Analysis, obtained from MAUVE output, green dots = poultry meat strains, red dots = human strains. One PM strain (C1-91) clusters with three H strains.

4.5 Discussion

E. coli strains belonging to phylogroups B2 and D are those most often associated with extra-intestinal infection in humans (Jaureguy et al., 2008, Manges and Johnson, 2012, Banerjee et al., 2013). Among the 724 distinct REP types detected in 237 *E. coli* positive meat samples 8.3% belonged to phylogroup B2 and 10.6% belonged to phylogroup D. The majority of phylogroup B2 and D strains isolated from humans represent a small number of sequence types. Indeed, the great majority belong to fewer than two-dozen clonal complexes, namely those represented by STs 12, 14, 73, 95, 127, 131, 141, 144, and 372 (all phylogroup B2), and ST69 (phylogroup D). However, most extra-intestinal isolates belong to just one of four well-defined clonal complexes (CCs), CCs 73, 95, 131, and 69, which are geographically widespread, if not cosmopolitan (Jaureguy et al., 2008, Manges and Johnson, 2012, Banerjee et al., 2013, Doumith et al., 2015, 2016).

Of the human associated STs belonging to phylogroup B2, only STs 95 and 131 were detected in poultry meat. ST95 (n = 17) accounted for almost half (46%) of phylogroup B2 isolates from poultry meat (n = 37) that were sequenced as part of this study. Representatives of all unique B2 isolates screened using REP-typing method was selected and sequenced. Other phylogroup B2 STs observed were ST135 (n = 11), ST131 (n = 6), ST355 (n = 2) and ST372 (n = 1). Comparisons of isolates from humans and poultry belonging to STs 135, 355 and 372 were not attempted, as these STs have not been detected in humans living in Canberra, Australia.

Fifteen (88%) of the ST95 strains from poultry belonged to a single ST95 subgroup characterised by O1:H7 *fimH27* strains. This subgroup represents 22% of the ST95 strains recovered from humans living in the Canberra region (unpublished data). The core genome phylogeny of human and poultry ST95 isolates suggests that there is little overlap of human and poultry meat isolates (Fig. 4.2a). Analysis of the variable gene content of these strains (Fig. 4.2b) suggests two clusters of strains; one cluster (n = 11) that represents mostly isolates from humans and another cluster (n = 12) that largely consists of isolates from poultry meat. There are 3 poultry meat isolates (25%) that have very similar variable gene content to the cluster isolates mostly recovered from humans.

This outcome suggests that the ST95 isolates recovered from these meat samples are likely to represent contamination of the poultry meat by human derived ST95 strains. It also appears that poultry meat ST95 isolates have succeeded in establishing in humans, as there are 3 strains from humans that have very similar variable gene content to the ST95 strains from poultry. The 'poultry meat derived' ST95 isolates found in humans represented 3 isolates from UTI.

All of the ST131 isolates from poultry represented O25B:H4 *fimH22* isolates. This group of ST131 strains represents 14% of the ST131 strains recovered from humans living in the Canberra region. However, the ST131 O25b:H4 *fimH22* isolates from poultry meat are clearly distinct from the O25b:H4 fimH22 isolates from humans (Fig. 4.3a, 4.3b).

Although the sample sizes for the phylogroup D ST69 isolates are small, the results indicate that ST69 strains from poultry are distinct from ST69 isolates from humans. There was one case of a poultry isolate exhibiting a variable gene content that was more similar to the isolates from humans than the poultry meat isolates (Fig. 4.4b), and this suggests contamination of the poultry meat sample by a human derived ST69 strains.

The analyses described above suggest that the contamination of poultry meat products by poultry derived strains is not a significant source of strains with the potential to cause extra-intestinal infections. Several of the most common STs recovered from humans (STs 73, 127, 12 and 14) were not detected in the poultry meat samples. Those human associated STs from poultry meat that were observed appear to be distinct from those recovered from humans. Indeed the results of this study might suggest that the consumption of poultry meat may expose a person to strains whose presence is a consequence of contamination of the meat product by human-derived strains. How much additional risk this represents is difficult to assess given the fact that strains associated with extra-intestinal infection are very likely to be shared among family members and their pets (Johnson et al., 2000, 2016).

Unlike STs 95, 131 and 69, that are common human associated strains, ST117 is infrequent among human isolates. There is no direct PCR-based screening method for ST117 and the isolates yield an F phylogenetic profile when using the quadruplex Clermont method (Clermont et al., 2013). In a collection of single isolates from each of 619 humans living in the Canberra region, 15 (2.4%) exhibited a phylogroup F profile (Gordon DM, unpublished data). MLST data for 15 of the phylogroup F isolates revealed that 3 isolates (23%) were actually ST117. Thus, in this collection of 619 *E. coli* isolates ST117 strains represented less than 1% of the *E. coli* isolated. Furthermore, ST117 was not observed among over 500 isolates of *E. coli* from native Australian vertebrates (unpublished data). ST117 has not been detected in native Australian birds nor has it been detected in backyard poultry in Australia (Blyton et al., 2015). By contrast, 15% of the poultry meat isolates yielded a phylogroup F profile and MLST results indicate that ST117 isolates represent about 10% of all poultry meat isolates. A number of other studies have also shown that ST117 strains represent a significant fraction of the *E. coli* recovered from poultry (Manges and Johnson, 2012, Bergeron et al., 2012, Mora et al., 2012, Maluta et al., 2014). If there is any real frequency data, add this data as well. Thus, ST117 appears to be an *E. coli* lineage largely associated with commercial poultry.

There appears to be less differentiation between ST117 isolates from poultry meat and from humans. There is no clear clustering of human or poultry meat isolates based on a core genome phylogeny (Fig. 4.1a). On average, there is a significant difference between human and poultry meat ST117 isolates based on their variable gene content (Fig. 4.1b, $p = 0.05$). The ST117 strains fall in one of three clusters, two of which consist entirely or large of poultry meat isolates, while the majority of isolates in the third cluster of ST117 strains are from humans.

None of the strains analysed in this study exhibited resistance to important broad spectrum antibiotics belonging to classes carbapenems (ertapenem), fluoroquinolones (ciprofloxacin), 3rd generation cephalosporins (ceftiofur, ceftazidime and cefotaxime) and very low resistance to 1st generation cephalosporin (cefazolin) and quinolone (nalidixic acid) (n = 2 each, all ST117). Overall, plasmid-mediated antimicrobial resistance

rates were also observed to be relatively low, with averages of 2.65 (ST117), 1.2 (ST95), 1.6 (ST131) and 0.3 (ST69), compared to other studies (Kluytmans et al., 2013, Johnson et al., 2012) which could be because of the strict regulations in use of antimicrobials, such as ban of fluoroquinolone use in food animals, and the ban of antimicrobial use as growth promoters for food animals in Australia (Collignon, 2015, Shaban et al., 2014).

Our findings suggest that there is a low rate potential transfer of zoonotic ExPEC and APEC lineages between humans and poultry meat, possibly happening in both directions i.e. human to poultry meat transfer and also poultry meat to human transfer in Canberra region. However, a larger scale sampling is required, starting from the farm level, right from the poultry birds including the feeds and water, then through the production chain including the processing plants and transportation of the meat products. The route of entry for these potential zoonotic *E. coli* can be linked to many different factors including (but not limited to) poor hygiene during processing and/or distribution, faecal contamination of carcasses through slaughtering and also initial colonization of the broilers even before processing, right from the farm level (Ghodousi et al., 2016).

4.6 Conclusion

The ability of ExPEC, APEC and intestinal pathogenic *E. coli* to cause diseases is associated with the presence of certain virulence-associated traits like toxins, adhesins, invasins, autotransporters, iron acquisition systems and other factors (Kaper et al., 2004). The presence of antimicrobial resistance genes, both chromosomal and plasmid-borne add to the complexity of treating the diseases caused by *E. coli*. This study indicates that poultry meat in Canberra region may serve as a low risk zoonotic reservoir or vehicle of ExPEC, specifically for ST95 and ST117 lineages. These strains harbour virulence genes that could aid in causing infections in both humans and poultry birds. Multi-drug resistance (MDR) was found to be relatively low and seen mostly in ST117 (41.2%), ST131 (33.3%) and ST69 (11.1%) but non-existent in ST95 poultry meat strains. Our findings highlights the importance of understanding and carrying out hygienic practices in poultry farms, production chains and food safety conditions need to be prioritized while handling and cooking poultry meat and other meats. Further studies

would be recommended to identify and trace the origin of contamination by sampling and tracing the meat sources right back from the broilers at the farm level, production chain including machines, human handlers and distribution chain which eventually reach retail supermarkets and butcheries.

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4.8 Supplemental Materials

Supplemental Table 4.1. Metadata of all strains with ST, serotype, plasmid replicon type (incompatibility group) and resistance profiles. The strains indicated in green = poultry meat strains and red = human strains.

Strain	ST	Inc Group	Serotype	Resistance genes
C4.34	95	FII	O1:H7	*tetB*
A1.66	95	FII	O1:H7	*tetB*
C3.27	95	FII, FIB	O1:H7	
W1.58	95	FII	O1:H7	*tetB*
W2.47	95	FII	O1:H7	*tetB*
W2.53	95	FII	O1:H7	*tetB*
W4.32	95	FII	O1:H7	*tetB*
W4.33	95	FII	O1:H7	*tetB*
W4.39	95	FII	O1:H7	*tetB*
C2.110	95	FII	H7	*tetB*
C2.117	95	FII	H7	*tetB*
C3.28	95	FII, FIB	H7	
C4.33	95	FII	H7	*tetB*
C4.35	95	FII	H7	*tetB*
W2.49	95	FII	H7	*tetB*

W4.35	95	FII	H7	*tetB*
W4.37	95	FII	H7	*tetB*
2H-276-5	95	FII	H7	*tetB*
62-2-TI6	95	FII	O1:H7	*tetB*
BS36	95	FII	O1:H7	*tetB*
M638866	95	FII	O1:H7	*tetB*
M644693	95		O1:H7	*tetB*
M652995	95	FII	H7	*tetB, tetA, blaTEM-1C*
M660875	95	FII	H7	*tetB*
M663475	95	FII	O1:H7	*tetB*
M663940	95	FII	O1:H7	*tetB*
M671335	95	FII	O1:H7	
M673438	95	FII	O1:H7	*tetB*
M710019	95	FII	O1:H7	*tetB, blaTEM-1C, sul2, strA, strB*
A1.49	131	I1, FII, FIB, FIC	O25:H4	*blaTEM-1B*
C2.116	131	I1, HI2, FII, FIB, FIC	O25:H4	*blaTEM-1B, tetA, sul1, dfrA12, aadA2*
C3.29	131	I1, HI2, FII, FIB, FIC	O25:H4	*blaTEM-1B, tetA, dfrA12*
C4.100	131	I1, FII, FIB, FIC	O25:H4	*blaTEM-1B*
W1.57	131	I1, FII, FIB, FIC	O25:H4	*blaTEM-1B*
W1.65	131	I1, FII, FIB, FIC	O25:H4	*blaTEM-1B*
55-1-AU4	131	FII, FIB	O25:H4	
BS003	131	FII, FIB, FIC	O25:H4	
M652394	131	FII, FIB, FIC	O25:H4	
M653835	131	FII, FIB	O25:H4	*blaTEM-1B, tetA, strB, sul1, dfrA7, catA1*
M683442	131		O25:H4	
M710059	131	FII, FIB	O25:H4	blaTEM-1C
C1.91	69		O17/O44:H18	*sul2, strA, strB*
C4.42	69	FII	O17/O44:H18	
W3.62	69	IncFII, FIB	O17/O44:H18	
22-1-Ti17	69		O17/O44:H18	
W2.76	69	FII, FIB, FIC	O17/O77:H18	*tetC*
W3.67	69	FII	O17/O77:H18	
11-1-Ti6	69		O17/O77:H18	
70-1-AC3	69	FII, FIB	O17/O77:H18	
W2.79	69		O21:H18	
A4.61	69		H49	
C2.45	69		H49	
W2.80	69		H49	
C2.100	117	FII, FIB	O111:H4	*tetB, dfrA5, sul1, sul2, strA, strB*
C2.102	117	FII, FIB	O111:H4	*tetB, dfrA5, sul1, sul2, strA, strB*
C2.103	117	FII, FIB	O119:H4	*sul2*
C4.85	117	FII, FIB	O143:H4	*tetA, dfrA5*
C3.72	117	I1, FII, FIB	O149:H10	*blaTEM-1B*
C1.123	117	FII, FIB	O161:H4	
C1.119	117	FIB	O45:H18	*blaTEM-1B, dfrA5, sul1*
C4.95	117	FIB	O45:H18	*blaTEM-1B, dfrA5*
C2.97	117	FII, FIB	O45:H4	*blaCMY-2*

C3.70	117	FII, FIB	O45:H4	*sul2, catA1, strA, strB*
C4.94	117	FII, FIB	O45:H4	*blaCMY-2*
C2.27	117	FII, FIB, FIC	O53:H4	*tetA, tetC*
C4.86	117	FII, FIB	O53:H4	
C2.106	117	FII, FIB	O71:H4	*sul2*
C4.93	117	FII, FIB	O71:H4	*sul2*
C2.13	117	FII, FIB	H4	*sul2*
C3.65	117	FII, FIB	H4	
103-1	117	FII, FIB	O143:H4	*blaTEM-1B, tetA, sul2, dfrA5, strA, strB*
290-1	117	I1, FII, FIB	H4	*blaTEM-1B, tetB*
434-21	117	FII, FIB	O111:H4	*tetB, dfrA5, sul1, sul2, strA, strB*
H030	117	FII, FIB	O114:H4	*blaTEM-1B, tetA, sul1, aadA5, dfrA17, oqxB*
H561	117	FII, FIB	O161:H4	
H299	117	FII, FIA, FIB, FIC	O24:H4	*blaTEM-1B, tetA, tetC, sul2, dfrA5, strA, strB*

Supplemental Table 4.2. Virulence factors distribution by STs and source.

Virulence factors	All strains (n=76) %	ST95		ST131		ST69		ST117	
		H (n=12) %	PM (n=17) %	H (n=6) %	PM (n=6) %	H (n=3) %	PM (n=9) %	H (n=6) %	PM (n=17) %
fimH	100	100	100	100	100	100	100	100	100
etsC	78.9	91.7	100	33.3	100	-	11.1	100	100
fyuA	78.9	100	100	100	100	33.3	11.1	100	64.7
hra	38.1	8.3	29.4	50	100	-	66.7	33.3	35.3
ibeA	15.8	-	-	100	100	-	-	-	-
ireA	65.8	100	100	-	-	-	-	100	88.2
iroN	71	91.7	94.1	33.3	100	-	22.2	100	64.7
iucC	92.1	91.7	100	83.3	100	33.3	88.9	83.3	100
iutA	93.4	91.7	100	83.3	100	33.3	88.9	100	100
kpsE	65.8	100	100	83.3	100	66.7	88.9	-	-
neuC	42.1	100	82.3	-	83.3	-	11.1	-	-
omp_chromo	93.4	100	100	100	100	66.7	55.5	100	100
ompT	81.6	91.7	100	33.3	100	-	33.3	100	100
papG	43.4	100	100	-	-	33.3	-	16.7	11.8
papC	47.4	100	100	33.3	16.7	33.3	-	16.7	11.8
sitA	96	91.7	100	83.3	100	100	100	83.3	100
terC	6.6	-	-	-	33.3	-	22.2	-	5.9
traT	82.9	91.7	100	83.3	100	33.3	44.4	83.3	82.3
usp	52.6	91.7	100	100	100	-	-	-	-
vat	72.4	100	94.1	-	50	-	11.1	100	100

cah_Ag43	77.6	100	100	50	100	66.7	100	16.7	52.9
cdiA	17.1	-	-	33.3	-	-	-	33.3	52.9
ybtS	78.9	100	100	100	100	33.3	11.1	100	64.7
ccl	2.6	-	-	-	-	-	-	-	11.8
iha	26.3	-	11.8	50	-	33.3	100	-	29.4
eaeH	100	100	100	100	100	100	100	100	100
tia	31.6	8.3	11.8	50	33.3	-	55.5	33.3	52.9
upaG	71	91.7	94.1	-	100	-	88.9	50	58.8
senB	5.3	-	-	50	-	33.3	-	-	-
tsh	19.7	-	11.8	16.7	100	-	11.1	16.7	23.5
arcA	56.6	100	88.2	100	100	-	-	-	23.5
astA	18.4	8.3	-	16.7	33.3	66.7	22.2	16.7	29.4
iss	93.4	100	94.1	83.3	100	66.7	77.8	100	100
gad	90.8	100	100	100	100	100	100	50	76.5
mchF	64.5	91.7	100	33.3	100	-	-	83.3	47
mchC	9.2	8.3	5.9	-	-	-	-	16.7	23.5
mchB	6.6	8.3	5.9	-	-	-	-	8.3	11.8
lpfA	47.9	-	-	-	-	100	100	100	100
celb	5.3	-	-	-	-	-	22.2	-	11.8
hlyE	13.1	-	-	-	-	-	-	50	41.2
pic	15.8							50	52.9
sat	5.3	-	-	50	-	33.3	-	-	-
mcmA	5.3	-	-	-	-	-	-	33.3	11.8
air	9.2	-	-	-	-	66.7	55.5	-	-
eilA	14.5	-	-	-	-	100	88.9	-	-
Col E1	55.3	91.7	88.2	16.7	-	-	77.8	83.3	17.6
Col Ia	59.2	91.7	100	16.7	100	-	-	50	41.2
Col Ib	15.8	16.7	-	-	-	-	-	50	41.2
Col B	7.9	-	-	-	-	-	44.4	-	11.8
Col M	15.8	-	-	-	-	-	55.5	33.3	29.4
Microcin V	63.1	91.7	100	33.3	100	-	-	83.3	41.2
Microcin B17	2.6	-	-	-	-	-	11.1	-	5.9
Microcin H47	69.7	91.7	100	33.3	100	-	11.1	100	58.8

Chapter 5

General Discussion

5.1 Overview of *E. coli in poultry meat*

The incidence of bacterial contamination in commercial poultry meat is common (Vogt et al., 2014, Jiménez et al., 2003). Poultry meat is frequently found to harbour the most 'human-like' *E. coli* isolates, as compared to other meats like beef and pork, capable of causing intestinal and extra-intestinal infections. The contamination source of common organisms like *E. coli* could range anywhere from the poultry itself (gut or faecal), farm environments, processing chains to transport, and even at retail levels. Also, the processing of poultry meat involves a number of steps and is considered to be far more complex than other meat types, thus further adding to probable contamination route (Jiménez et al., 2003, Pacholewicz et al., 2015). Therefore, the prevalence and abundance of *E. coli* in poultry meat products may not necessarily indicate faecal contamination as there are several factors that could serve as routes of cross-contamination in the production and distribution chain.

In this study, out of 306 meat samples collected, *E. coli* was isolated in 77.5% (n=237) of the meat samples, mostly by enrichment and were detected at low cell densities. In total, 3415 *E. coli* isolates were recovered using two enrichment broths, namely lauryl sulfate (LS) broth and lysogeny (LB) broth supplemented with Vancomycin. Antibiotic selection method was also used to recover isolates present in the meat samples. Enrichment was carried out for maximizing *E. coli* isolation to better study the population diversity of *E. coli* in poultry meat. Out of the 3415 isolates recovered and characterized, 724 strains with unique REP-type or profile were identified. Phylogroup A was the most prevalent (27.2%), followed closely by B1 (21.3%), and then to lesser proportions phylogroups E (16.4%), F (15.2%), D (10.6%), and B2 (8.3%) strains.

The high diversity of *E. coli* isolates seen in this study is probably not due to "generalised environmental contamination". Relatively few STs were observed out of representative strains, and majority of the isolates were often detected more than once from multiple meat types, stores, supermarkets, and even seasons. Isolates belonging to phylogroup A were over-represented, thus indicating that a lot of the strains present in commercial poultry meat are most likely poultry-associated *E. coli* rather than human-associated *E.*

coli (this study, Obeng et al., 2012). In Australia, phylogroup B2 isolates are frequently over-represented in *E. coli* isolated from human sources (both clinical and commensal) while they were the least commonly isolated phylogroup in poultry meat samples, among the major phylogroups A, B1, B2 and D (Gordon et al, 2015, Blyton et al., 2014, this study). This further indicates that the major strains contaminating poultry meat come from the birds which belong to poultry-associated groups (like phylogroup A) rather than from humans.

Of note, the Clermont phylogroup profiles assigned to the isolates using Clermont et al. (2013) method was not always accurate, after allocating them to phylogenetic trees using whole genome sequences of representative strains. Four strains typed as phylogroup A1 clustered with phylogroup C in the phylogenetic tree inferred, 5 strains typed as E strains belonged to Clade I, and 27 phylogroup D strains clustered under phylogroup A. Also, 2 phylogroup D strains were assigned as phylogroup E, and 7 strains that were originally assigned "unknown" as they exhibited a profile undefined by the new Clermont quadruplex method (*arpA*, *yjaA*, *tsp* positive), also clustered under phylogroup A in the phylogenetic tree inferred. Genomic rearrangements like gene gain/ loss events is a common occurrence in bacteria like *E. coli* with high genome plasticity (Touchon et al., 2009, Tenaillon et al., 2010), which can thus affect the phylogroup misassignments. This indicates that *E. coli* strains in commercial poultry have evolved, which is also demonstrated in the high serotype diversity within strains of same STs (for example, 10 different serotypes out of 17 ST117 strains).

Among these "evolved strains", novel STs (ST6047 and ST6053) were identified, which had high VFs content (average of 18.8 and 20.9 respectively; Chapter 3). Furthermore, all 8 ST6053 strains were MDR, harbouring plasmid-mediated resistance genes to four different antimicrobial classes [penicillin (ampicillin, *blaTEM-1B*), sulfamethoxazole (*sul1*, *sul2*), trimethoprim (*dfrA5*), and aminoglycoside (streptomycin, *strA*, *strB*)]. Even though the strains were classified as phylogroup D1 using the new Clermont quadruplex method (*arpA*, *chuA* positive), they actually clustered to phylogroup A when the phylogenetic trees were inferred using WGS data. The strains were isolated across all

three seasons (summer, autumn, winter), from all three supermarkets (and two butchers in Belconnen and Tuggeranong), and in all four town centres (except Woden for ST6053). This proves the successful dissemination of these strains, which so far have never been detected in other hosts including humans, wild birds, and environmental samples (like water).

The presence of several REP-types or strains in a single meat sample, say breast meat, indicated the heterogeneity of *E. coli* population in poultry meat. It further proves the diverse heterogeneous nature of *E. coli* and also points to ongoing evolution. The gut bacteria turnover of commercial poultry is likely to be high because of several factors like housing, feed and water (Gordon DM, personal communication). High densities of birds are placed in close proximity as flocks, thus giving rise to plenty of chance for among-host transmission of *E. coli* to occur. Consequently, the chance of clonal dissemination of strains may be high where strains are spread in entire flocks, although this could potentially be contained because of high turnover of birds. The rearing and processing of birds in commercial poultry farms are carried out in batches, where entire flocks are reared for equal durations and then sent off for processing (35-55 days approximately for conventional and free range birds, and 65-80 days for organic meat birds). Thus, even though certain lineages/ clonal groups of *E. coli* might have disseminated successfully in one batch of chickens, they may not necessarily be present by the time the next batch of birds arrive the farm.

5.2 Risks associated with poultry meat *E. coli*

Poultry meat as a potential reservoir of intestinal and extra-intestinal infection causing strains, in both humans and poultry, has been a focus of many studies worldwide (Manges, 2016, Ewers et al., 2009, Johnson et al., 2008a, Chinen et al., 2009). Although undercooked meat as a source of diarrheal infections is well-established, its role as a source of opportunistic zoonotic pathogens like ExPEC capable of causing infections like UTI, septicemia, and other extra-intestinal infections, is not always straightforward (Manges, 2016). Several studies have characterized identical strains (using PFGE and RAPD techniques) from poultry meat and human ExPEC infection sources (Jakobsen et

al., 2012, Manges et al., 2007, Johnson et al., 2005a). In our study, a link between human ExPEC strains and poultry meat was established, but at very low levels (Chapter 4).

In addition, poultry meat has often been associated to harbour higher resistance determinants than other meat products like beef and pork (Sheikh et al., 2012, Vogt et al., 2014, Johnson et al., 2009, Vincent et al., 2010, Overdevest et al., 2011). In this study, phenotypic resistance was detected against 11 out of 13 tested antimicrobial classes, commonly to tetracycline (39%), ampicillin (27.4%), and to a lesser extent, sulfamethoxazole-trimethoprim (16.8%). Out of the 283 strains sequenced, 64% (n=181) harboured either or both chromosomal and plasmid-mediated resistance determinants. These resistance rates and patterns observed are often different (lower) to those detected by other research groups in different countries like Netherlands (Overdevest et al., 2011), Canada (Sheikh et al., 2012), United States (Johnson et al., 2009) and China (Wu et al., 2014).

Unlike several studies that use traditional molecular typing methods like MLST, VF content, and phylogroup membership for comparison studies to determine relatedness (Aslam et al., 2014, Manges et al., 2015, Vincent et al., 2010), we used a whole genome-based approach. These studies provided strong evidence that a food-animal reservoir of ExPEC strains in the form of poultry meat was present and the overlap between the two sources was quite significant. In our study, although we identified common ExPEC-related lineages (like ST131 = 6, ST95 = 17, ST117 = 17, ST69 = 9) in poultry meat, the prevalence was not high. Furthermore, when extensive gene content comparisons were carried out with these lineages, it was evident that the human strains and poultry meat strains clustered quite distinctly separate, particularly for ST131 and ST69 strains. Our findings suggest that it is unlikely that human ST131 and ST69 isolates disseminated from poultry meat, nor that poultry meat is contaminated with human strains of these lineages. However, this does not undermine the fact that these strains harboured high numbers of VFs, commonly carried on mobile genetic elements (plasmids), especially ST131 strains. Therefore, the presence of these lineages in poultry meat suggests that

they could still potentially serve as ExPEC reservoirs even if a direct transmission link to/ from humans may not be established.

However, the presence of ST117 strains, a poultry-associated lineage in humans indicate potential transfer from poultry sources to humans, although the route of entry is hard to determine (Maluta et al., 2014a). The strains could have been introduced to humans not only through consumption but also potentially by cross-contamination during handling. These strains harboured VFs commonly associated with APEC infections like *vat*, *cva*, *iss*, *irp2* (Maluta et al., 2012, 2014a) and also, ExPEC-related VFs like *sitA*, *ireA*, *iutA*, *traT* (Johnson et al., 2005a, b). Also, the APEC-specific hemolysin gene *hlyE* was detected only in ST117 strains, 7 poultry meat and 3 human strains which further suggests probable dissemination and transfer of APEC-related virulence mechanisms in both meat and humans.

The clonal group ST95 is linked to both human ExPEC lineage (Johnson et al., 2008a) and poultry APEC lineage (Maluta et al., 2014a, Ewers et al., 2009). The lineage is often over-represented in UPEC and NMEC isolates which are responsible for causing human UTI and neonatal meningitis respectively, and additionally from colibacillosis infection sites in birds (Johnson et al., 2008a, Ewers et al., 2009, Vincent et al., 2010, Maluta et al., 2014a). A number of studies also conducted animal experiments where APEC strains were capable of causing infections in the animal models studied (Maluta et al., 2014b, Moulin-Schouleur et al., 2007, Ewers et al., 2009). ST95 was over-represented among phylogroup B2 isolates that were isolated from poultry meat (45.9%), although it accounted for only 18% among human isolates. From our gene content comparison study, our findings indicate that humans are more likely to acquire poultry-associated ST95 (33.3%) than poultry meat being contaminated with human-associated ST95 strains (16.7%). Consequently, cross-contamination between the two sources is probable, and similar to other findings worldwide, the intestine of poultry most likely serves as a reservoir of APEC strains (Ewers et al., 2009, Maluta et al., 2014a, McPeake et al., 2005).

Although the presence of ExPEC and APEC-associated strains in poultry meat can be deemed as a substantial zoonosis reservoir, it does not mean that these strains come from infected humans and birds. Healthy humans and birds can serve as reservoirs of these opportunistic strains as indicated in several studies (Gordon et al., 2015, Blyton et al., 2014, Solà-Ginés et al., 2015). Consequently, unless identical strains have been isolated from infection sites in diseased hosts, it cannot be confirmed that the ExPEC and APEC-associated isolates from poultry meat are a result of contamination from sick humans and birds.

Intestinal/ diarrheal pathogenic *E. coli* (IPEC) and ExPEC isolates often harbour VF genes quite distinctly different from each other. For instance, VFs like intimin genes (*eaeA, tir*) and Shiga-toxin genes (*stx*$_1$, *stx*$_2$) are IPEC-specific genes, while fimbriae genes (*sfa*, *foc*) and adhesin genes (*draA*, *afa*) are specific for ExPEC strains (Kaper et al., 2004). In this study, only two out of 283 strains analysed harboured the IPEC sub-type EPEC/ EHEC-specific genes like *eaeA, espA/B, cif, tir,* and *nleB*. Both strains belonged to phylogroup A and were of sequence type (ST) ST10, the lineage often associated with diarrheal infections (Okeke et al., 2010).

On the other hand, strains harbouring ExPEC-associated VFs were more common. Out of 283 strains, 55 strains (19.4%) had ExPEC status as per Johnson et al. (2003), thus harboured two or more of the genes *sfa/ focDE*, *afa/ draBC*, *papA/ C*, *iutA*, and *kpsMT* II (Smith et al., 2007). The over-represented human ExPEC-related genes that were detected were *traT, sitA*, *ibeA*, *ireA*. Additionally, APEC-associated genes as per Johnson et al. (2008b) like *cva*, *tsh*, *iss* were also identified (28.3%), suggesting poultry meat as a potential APEC reservoir similar to other studies (Johnson et al., 2008a, Maluta et al., 2014a).

The strains with high VFs content predominantly belonged to phylogroups B2, E, D and Clade I, which are the phylogroups commonly associated with ExPEC and APEC strains (Manges and Johnson, 2015, Solà-Ginés et al., 2015), with the exception of phylogroup E. From our findings, phylogroup E was more likely to harbour APEC-related genes

compared to other phylogroups. The clonal groups with high VFs content (like B2-ST131, B2-ST95, F-ST117, C-ST88, Clade I-ST770, and E-CC350) in our study have also been identified in other studies suggesting a successful clonal dissemination (Platell et al., 2011, Skurnik et al., 2016, Manges et al., 2015, Maluta et al., 2014a). It is noteworthy that some lineages associated with high VFs content (like ST95, ST770) were less likely to exhibit multi-drug resistance, although this pattern was not always the case, as seen in clonal groups like ST117 and CC350 (ST57) which were often MDR. In general, commensal groups of phylogroups B1 and A were less likely to harbour high VFs, while common ExPEC lineages of phylogroups B2, D and F were more likely to exhibit higher VFs content.

Of note, the rates of resistance detected against critically important antimicrobials (Collignon et al., 2016) like fluoroquinolones and 3rd generation cephalosporins in Australia is much lower than that in other countries like the United States (Johnson et al., 2006, 2005a, b), Greece (Gousia et al., 2011), Switzerland (Vogt et al., 2014), Spain (Solà-Ginés et al., 2015) and China (Wu et al., 2009, 2014). This can be linked to the ban on use of these antimicrobial classes in food-producing animals in Australia (Collignon, 2015, Cheng et al., 2012), while fluoroquinolones (enrofloxacin) and 3rd generation cephalosporins are still used in some countries (Collignon et al., 2013), not only as therapeutics but also as metaphylaxis and growth promoters (Gousia et al., 2011, Krishnasamy et al., 2015). Fluoroquinolone (ciprofloxacin) resistance in *E. coli* was detected in a clonal group CC354 of phylogroup F, that were also isolated in companion animals like dogs (Guo et al., 2015), backyard poultry and native birds (Blyton et al., 2015), and humans in Australia (Vangchhia et al., 2016), indicating successful dissemination of this lineage in multiple hosts. Additionally, three poultry meat strains harboured the gene *blaCMY-2*, the plasmid-mediated resistance determinant gene of 3rd generation cephalosporin (ceftazidime). The effect of the presence of these antimicrobial-resistant strains in poultry meat on human health, even if low (1.8% for fluoroquinolone, and 0.9% for 3rd generation cephalosporin), is still significant and indicates cross-contamination of meat samples rather than off-label use in the poultry industry (Vangchhia et al., 2016).

Australia is also in a unique position of solely producing poultry meat products domestically with no international imports, which significantly helps in curbing the development of antimicrobial resistance especially to critically important classes which are not approved nor administered for use in the country (ACMF, 2016). Countries like Denmark (Hasman et al., 2015), Switzerland (Zogg et al., 2016) and Netherlands (Kluytmans et al., 2016) have reported the presence of *E. coli* strains resistant to even the last line antimicrobial classes like colistin and carbapenems in imported poultry meat products. Also, China (Shen et al., 2016), United Kingdom (Doumith et al., 2016), are also already detecting these multidrug-resistant (MDR) strains not only in food-producing animals but also in meat products. To the best of our knowledge, resistance to carbapenems and colistin has so far not been detected in foodborne *E. coli* in Australia, including in this study. It is noteworthy that the judicious use of antimicrobials in commercial poultry farms, and restricting the import of poultry meat for human consumption seem to have a significant impact on the low levels of antimicrobial resistance (AMR), at least to the classes that are banned for use in food-producing animals (Collignon, 2015). Therefore, poultry meat and commercial poultry may not serve as a major dissemination source and origin for antimicrobial resistance in Australia.

5.3 Commercial poultry versus backyard poultry

E. coli forms part of the normal gut flora of poultry, regardless of their rearing methods, whether they are raised in commercial production farms or as domesticated chooks in backyards of private homes (Blyton et al., 2015, Kemmett et al., 2013, Ewers et al., 2009). The birds are often associated to harbour highly diverse *E. coli* populations based on different study findings, usually conducted by means of faecal sampling procedures (Kemmett et al., 2013, Solà-Ginés et al., 2015). Phylogroup A is commonly over-represented in the faeces of commercial poultry (Obeng et al., 2012, Kemmett et al., 2013, Solà-Ginés et al., 2015), and also on domesticated poultry (Escobar-Páramo et al., 2006) similar to our findings (Blyton et al., 2015) in backyard poultry. Interestingly, our study is consistent with several other studies, where, in poultry meat products, phylogroup A are also known to be present in higher proportions than the other

phylogroups (this study, Aslam et al., 2014, Johnson et al., 2007, Johnson et al., 2009, Jakobsen et al., 2010). Although the higher prevalence of the same phylogroup in both faecal samples of meat chickens and meat products could potentially indicate faecal contamination of meat samples, it does not necessarily confirm this hypothesis. Phylogroup A is associated to have broad host-spectrum (Gordon and Cowling, 2003), and have also been implicated in APEC isolates recovered from infection sites in poultry (Rodriguez-Siek et al., 2005).

Backyard poultry has also been linked as a reservoir of AMR *E. coli* in Australia (Blyton et al., 2015) and other countries (Nakayama et al., 2016, Pohjola et al., 2016, Braykov et al., 2016). High amounts and off-label use of antibiotics in domesticated birds and backyard poultry, especially to classes not approved for use in commercial poultry industries (for example, fluoroquinolones), is determined as one of the factors responsible for prevalence of MDR bacteria like *E. coli* (Blyton et al., 2015, Nakayama et al., 2016). The high prevalence of tetracycline resistance in both backyard and commercial poultry is an indication that the resistance genes are circulating from the birds rather than through human contamination, as tetracycline is not used in humans in Australia (Blyton et al., 2015). On the other hand, tetracycline is largely used for treating (therapeutic) and preventing outbreaks (prophylactic) in poultry (Shaban et al., 2014, Barton and Wilkins, 2001). Therefore, the prevalence of resistance to certain antimicrobials like tetracycline and ampicillin can certainly be attributed to the widespread use of the same antimicrobial classes (Collignon, 2015). Although resistance to these antimicrobials not used in humans may not pose an immediate threat to humans as such, it will eventually be a problem for poultry industries and public health, as they will soon fail to work in treating sick birds (Barton and Wilkins, 2001). This, in turn, will give rise for the need to use broad-spectrum antibiotics which are important and used in humans.

5.4 Conclusion

With poultry meat being the most consumed meat in Australia, it is important to know what exactly contaminates the meat that we eat, as often indicated "we are what we eat" (Collignon, 2009). The meat has often been associated to harbour the most "human-like" *E. coli*, which are often multi-drug resistant and virulent, capable of causing intestinal and extra-intestinal infections (Manges, 2016). Based on our findings, we conclude that poultry meat, though serving as a potential reservoir, is not the most significant reservoir of ExPEC and APEC-related strains in Canberra region. Even though genetically similar strains are circulating in humans and poultry meat, these strains do not appear to be identical and there is little intermingling between the two sources. Therefore, poultry meat most likely serves as a low-risk foodborne source of zoonotic *E. coli* through consumption and handling.

Moreover, our findings suggest that evolution of *E. coli* in commercial poultry meat is likely to be different from what occurs in human strains. Commercial poultry hence serves as an indicator of high evolution in *E. coli* isolates and also suggesting that the evolution is ongoing, within the highly heterogeneous *E. coli* population. This also confirms that poultry meat is more likely to be contaminated with *E. coli* strains from the birds rather than from humans.

Additionally, it is unlikely that poultry meat serves as the main dissemination source/ route of antimicrobial resistant *E. coli*. However, the presence of plasmid-mediated resistance genes which can easily be disseminated and transferred from poultry meat to other sources is a concern. Furthermore, the presence of certain multi-drug resistant isolates can neither be undermined nor the detection of strains that are resistant to antibiotics critically important to human health (like fluoroquinolones and 3rd generation cephalosporins). In fact, the finding of resistant isolates, which may not be as high as in other countries, balances to the fact that millions (>600 million) of poultry are reared and slaughtered for human consumption. And if taken on an average, the significance of resistance can still be highly exaggerated. These factors are enough to

elaborate the importance of characterizing the bacterial strains present in the meat we consume, both for awareness and health.

5.5 Future directions

The extensive study on *E. coli* in poultry meat conducted for this project is limited in a number of ways. It is hard to determine and conclude from our findings the point source where contamination is coming from, although this may pose a challenge regardless of extensive studies because there can be so many sources where the contamination can originate from (Northcutt et al., 2012). The ideal sampling design would include tracing samples from the farm level through the processing plants, transport chains and eventually leading to retail level distribution chains. The farm level sampling can include (but not limited to) faecal and gut microbial populations of both breeder birds and birds reared for commercial meat chicken, the birds' feeds and water, bedding, housing and the other environments they are exposed to including soil from the farm vicinities. Conducting sampling in between different processing steps including bleeding, scalding, de-feathering, evisceration, and chilling of chicken carcasses will also aid in providing a thorough microbial content check on the meat samples (Pacholewicz et al., 2015).

In addition, animal model experiments for testing the pathogenicity and/ or lethality of the *E. coli* strains with high virulence factors would be ideal to understand and/ or confirm pathogenesis of strains (Moulin-Schouleur et al., 2007). Animal model experiments could also be used to understand potential zoonotic lineages (like ST117) better, especially with regards to how well they are capable of adapting to the hosts' environment, under controlled/ monitored experimental settings like diet, temperature.

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Appendix

Phylogenetic Diversity, Antimicrobial Susceptibility and Virulence Characteristics of Phylogroup F *Escherichia coli* in Australia

Phylogenetic Diversity, Antimicrobial Susceptibility and Virulence Characteristics of Phylogroup F *Escherichia coli* in Australia

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Running title. Clinical characteristics of phylogroup F *E. coli*.

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Unlike *Escherichia coli* strains belonging to phylogroup B2, the clinical significance of strains belonging to phylogroup F is not well understood. Here we report on a collection of phylogroup F strains recovered in Australia from faeces and extra-intestinal sites from humans, companion animals, and native animals, as well as from poultry meat and water samples. The distribution of sequence types was clearly non-random with respect to isolate source. The antimicrobial resistance and virulence trait profiles also varied with the sequence type of the isolate. Phylogroup F strains tended to lack the virulence traits typically associated with phlyogroup B2 strains responsible for extra-intestinal infection in humans. Resistance to fluoroquinolones and/or expanded-spectrum cephalosporins was common within ST648, ST354, and ST3711. Although ST354 and ST3711 are part of the same clonal complex, the ST3711 isolates were only recovered from native birds being cared for in a single wildlife rehabilitation centre, whereas the ST354 isolates were from faeces and extra-intestinal sites of dogs and humans, and from poultry meat. Although ST354 isolates from chicken meat in Western Australia were distinct from all other ST354 isolates, those from poultry meat samples collected in eastern Australia shared many similarities with other ST354 isolates from humans and companion animals.

Introduction

It is well established that *Escherichia coli* exhibits extensive genetic structure and that strains of the species can be classified into four major and four minor phylogenetic groups (phylogroups). The great majority of *E. coli* isolates belong to the phylogroups known as A, B1, B2, and D. Strains of these phylogroups vary in their phenotypic and genotypic characteristics, ecological niche, lifestyle, and propensity to cause disease (Tenaillon et al., 2010). The minor phylogroups are known as C, E, F, and cryptic clade I (Clermont et al., 2013). Although these minor phylogroups have been recognised for several years, little is known about their geographic distribution, host preferences, phenotypic and genotypic characteristics, or propensity to cause disease. Until recently, the only way of identifying strains belonging to the minor phylogroups was by multi-locus sequence typing (MLST); this is the main reason why so little is known about the nature of these strains, as MLST characterisation of large strain collections is often prohibitively expensive. However, Clermont and colleagues (2013) described an improvement to the classic triplex PCR method for phylogroup assignment (Clermont et al., 2000) that enables isolates belonging to the minor phylogroups to be identified.

Of the minor phylogroups, strains belonging to phylogroup F are of particular significance as they have been implicated as extra-intestinal pathogens of companion animals (Guo et al., 2015), horses (Ewers et al., 2014), cattle (Abraham et al., 2015), and humans (Lau et al., 2008). Phylogroup F strains have also been found at high frequency in the faeces of wild birds being treated in wildlife rehabilitation centres (Blyton et al., 2015). Further, many phylogroup F clinical isolates are resistant to fluoroquinolones and/or expanded-spectrum cephalosporins.

The clinical significance of phylogroup F strains as causative agents of extra-intestinal infection and carriers of antimicrobial resistance determinants led us to examine phylogroup F strains isolated in Australia from extra-intestinal sites and faeces from a variety of host species, and from poultry meat and water samples. The strains were characterised for their antimicrobial resistance phenotype and, through whole genome sequencing, their virulence gene profiles and phylogenetic relationships. A more

detailed analysis of clonal complex CC354 was undertaken, as strains from this complex are frequently isolated from animals in clinical settings in Australia (Guo et al., 2015), are typically fluoroquinolone-resistant (Guo et al., 2015), and have been isolated from poultry meat products destined for human consumption (Ingram et al., 2013).

Materials and Methods

Strains

The 87 strains examined here represent an *ad hoc* collection of published and newly obtained clinical, commensal, environmental, and poultry meat isolates from diverse locales that were selected to represent the diversity of phylogroup F in Australia (Table 1). Also included were two NCBI reference strains (SMS-3-5 and IAI39) and several other isolates from non-Australian localities for which whole genome sequence data was available (Supplemental Table 1). The collection included 47 isolates recovered without antibiotic selection from the faeces of native Australian birds (Blyton et al., 2015) and mammals (Gordon and Cowling, 2003), from water samples (Power et al., 2005), as well as from humans living in Australia (Gordon et al., 2005; Blyton et al., 2014; Gordon et al., 2015). In addition the collection included 18 fluoroquinolone (FQ)-resistant isolates recovered either from dog faeces (Guo et al., 2015) or extra-intestinal isolates from humans (Turnidge et al., 2014) and dogs (Guo et al., 2015). As well, the collection included eight FQ resistant isolates recovered from poultry meat samples collected in Western Australia (Ingram et al., 2013) and nine FQ resistant isolates recovered from chicken meat products purchased from different retail outlets in Canberra, Australian Capital Territory (unpublished data). The basic metadata for all the phylogroup F strains examined here is presented in the Supplemental File.

DNA extraction and whole genome sequencing

Using ISOLATE II Genomic DNA kits (Bioline), genomic DNA was extracted from a 100 µL aliquot of a 5 ml lysogeny broth culture after overnight incubation at 35° with shaking (150 rpm). Genomic DNA was quantified using Qubit dsDNA BR assay kit (Invitrogen). Libraries were prepared using the Nextera XT DNA sample preparation kit (Illumina) and

the Nextera XT index kit (Illumina), with 0.5 ng of input DNA, according to the manufacturer's protocol.

Whole genome DNA sequencing was performed on an Illumina MiSeq platform using a 600-cycle MiSeq Nextera XT version 3-reagent kit (2x300 paired-end reads). The raw genomic sequencing data files were assembled as *de novo* genome sequences and exported as fasta files using CLC Genomics Workbench 8. The assembled data for all strains are available in Enterobase (http://enterobase.warwick.ac.uk/).

In silico characterisation

The strains were assigned to sequence types (STs) for the University of Warwick MLST scheme (http://enterobase.warwick.ac.uk/). The Centre for Genomic Epidemiology (CGE) website (www.genomicepidemiology.org) was used to characterize the strains using the VirulenceFinder, ResFinder, SeroTypeFinder, and PlasmidFinder tools. The presence of additional extra-intestinal virulence factors, beyond those detected by VirulenceFinder, were determined using CLC Genomics Workbench.

Comparative genomics

Phylogenetic relationships among the study isolates were inferred by aligning the strains to the reference phylogroup F strain SMS-3-5 using the HARVEST suite of tools (Treangen et al., 2014). Single-nucleotide polymorphisms (SNPs) were extracted and were used to construct a phylogeny using PhyML (Guindon et al., 2010), together with a general time reversible model of evolution. For CC354 strains the alignment program MAUVE (Darling et al., 2010) was used to determine the variable gene content which was extracted from the MAUVE backbone file. DNA regions smaller than 100 bp were removed, as were regions present in all strains or only a single strain. The remaining regions were scored as being either present or absent.

For the CC354 isolates, the gene content of the plasmids harbouring resistance gene determinants was inferred using an iterative process. First, the assembly contigs containing antimicrobial resistance genes were identified, as were contigs containing other known plasmid-borne genes (example: colicin B, *iroN*). The plasmid-associated contigs were then joined into a plasmid assembly for each isolate and the plasmid assemblies were aligned with the full assemblies of all CC354 isolates. Additional plasmid-associated contigs were identified, and the process was repeated until no new plasmid-associated contigs could be discovered. The resulting inferred plasmid assemblies were compared against the NCBI database by using blast to confirm that the resulting plasmid assemblies did not contain genes normally associated with the chromosome. The plasmid assemblies (contigs) for each strain were then aligned using MAUVE and the variable gene content of each strain was extracted from the backbone file.

Antimicrobial susceptibility testing

Susceptibility to clinically relevant antimicrobials was assessed by disk diffusion method on Mueller-Hinton agar plates (Acumedia, Neogen) as described by the European Committee on Antimicrobial Susceptibility Testing, and interpreted according to their published breakpoints (EUCAST, 2013). The antimicrobials represented different classes, namely, penicillins (ampicillin- 10 mcg, amoxicillin-clavulanic acid- 20/10 mcg), quinolones (nalidixic acid- 30 mcg), fluoroquinolones (ciprofloxacin- 5 mcg), aminoglycosides (gentamicin- 10 mcg), nitrofurans (nitrofurantoin- 100 mcg), carbapenems (ertapenem- 10 mcg), sulfonamides (trimethoprim-sulfamethoxazole- 23.75/1.25 mcg), tetracyclines (tetracycline- 30 mcg), 1st-generation cephalosporins (cefazolin- 30 mcg), and 3rd-generation cephalosporins (cefotaxime- 30 mcg, ceftazidime- 30 mcg, ceftiofur- 30 mcg) (Becton, Dickinson and Company). Inhibition zone diameters were measured using ProtoCOL 3 (Synbiosis).

Results

Group F strain phylogenetic relationships

The assemblies of the 87 group F strains were aligned using SMS-3-5 as the genome reference strain. The analysis revealed 119,524 SNPs in the core genome of these strains; these were used to infer the phylogenetic relationships among the strains (Fig. 1). The 87 strains represented 21 STs, with ST354 represented by 27 isolates, and STs 59 and 3711 represented by 12 isolates each.

The distribution of STs by source was clearly non-random. Only three ST354 isolates were from humans, while all ST59 and ST62 isolates (except strain TA326) were from humans. Similarly, all ST648 strains but one (30 1 R8) were extra-intestinal isolates from humans or companion animals, while all ST3711 isolates were recovered from native birds at one Western Australia locality.

Serotype Diversity

By using an *in silico* approach, 49% of the 87 isolates could be assigned an O type (Supplemental File). Ten O types were identified, with O1 being most common (20%), followed by O8 (9%) and O11 (6%). In contrast, all isolates could be assigned an H type. Eleven H types were identified, with H34 being most common (57%), followed by H7 (17%).

Virulence Determinants

In silico screening revealed that, overall, the phylogroup F isolates tended not to encode virulence factors implicated in extra-intestinal infection (Supplemental File). That is, no F isolates contained *clbB*, *focG*, *sfaA*, *lpfA*, *tcpC*, or *vat*, and very few harboured *afaD* (4%), *etsC* (3%), *hlyD* (1%), *ireA* (6%), *iroN* (2%), *terC* (1%), *cdtB* (2%), or *tsh* (1%). Virulence genes present in > 10% of isolates included *fyuA* (32%), *hra* (23%), *ibeA* (39%), *iutA* (59%), *neuC* (13%), *ompT* (37%), *papC* (17%), *sitA* (79%), *traT* (53%), *usp* (64%), *cah* (70%), *iha* (45%), *tia* (23%), *upaG* (11%), and *senB* (13%). It has been suggested that if

an *E. coli* strain possesses two or more of the traits *papAH*, *afa/draBC*, *sfa/focDE*, *kpsMT*.II or *iutA* it is capable of causing a urinary tract infection (Johnson et al., 2003). Only 13 (11%) of the phylogroup F strains met this criterion, and all but two of these (DAEC9 and GNB211) are members of STs 62 or 59.

The presence of many virulence traits varied by ST. For example, five traits (*fyuA*, *ibeA*, *iutA*, *usp*, and *iha*) tended to be uniformly present or absent in all representatives of a given ST. Likewise, the number of virulence factors detected per isolate varied by ST ($P < .001$, Kruskal-Wallis test). That is, for the seven STs represented by more than three isolates each, the average number of virulence factors per isolate was: 7.0 (ST3637), 8.0 (ST3711), 11.0 (ST354 and ST648), 13.7 (ST62), and 16.2 (ST59).

Bacteriocins

Bacteriocin genes were detected in 39 of the isolates, with colicin genes seen in 35 isolates and microcin genes in eight (Supplemental File). Only five isolates carried multiple bacteriocin genes. The various bacteriocin genes differed significantly for overall prevalence and distribution by ST. That is, colicin E1, the most common bacteriocin (23% of isolates overall), was restricted to ST354 and ST59. Colicin M, the next-most common bacteriocin (17% of isolates overall), was present in all ST3711 isolates. In contrast, microcin B17 was detected in only 6.9% of isolates (ST59), microcin H47 in 3.4%, and colicins B and Ia/Ib in one isolate each. Microcin (colicin) V was not detected.

Antimicrobial resistance phenotypes and determinants

The susceptibility results must be interpreted in relation to the methods used to obtain the isolates, as summarised here. Most of the canine faecal and clinical isolates, and all human clinical isolates, were selected originally because they were fluoroquinolone-resistant. Antimicrobial selection also was used to recover resistant isolates from poultry meat. In contrast, no antimicrobial selection was used in isolating faecal or biopsy strains from humans in the Canberra region or from birds and native mammals.

Susceptibility patterns among isolates varied greatly by source and ST (Supplemental File). For example, by source, most human faecal isolates from the Canberra region were susceptible to all tested antimicrobials, whereas, all avian faecal isolates from Western Australia were FQ-resistant. Likewise, by ST, most ST354, ST648, and ST3711 isolates were FQ-resistant, while ST59 and ST62 isolates were FQ-susceptible. On average, and irrespective of source, FQ-resistant isolates were resistant to four other antimicrobial classes, while FQ-susceptible isolates were resistant to ≤ 1 antimicrobial class ($P < 0.001$, Mann-Whitney test).

The number of antimicrobial resistance determinants detected by whole-genome sequencing also varied by ST ($P < 0.001$, Kruskal-Wallis test(Supplemental Table Supplementary data). Resistance determinants were absent in ST3637, and were uncommon (< 2 per isolate) in ST457, ST59, and ST62. In contrast, the average number of resistance determinants per isolate was 5.5 for ST354 and ST3711, and 9.2 for ST648.

Characteristics of CC354

Almost half of the study isolates represented two closely related STs within phylogroup group F, ST354 and ST3711. These two STs differed by a single MLST locus and hence were members of the same clonal complex (CC), CC354. Because these isolates were all FQ-resistant, this CC was characterised more fully.

After alignment of the ST354 and ST3711 isolates' genomes using MAUVE and identification of the core genome, variable positions in the core genome were extracted and used to infer a phylogeny (Fig 2). In this phylogeny, all ST3711 strains were closely related, while the ST354 strains exhibited some subclustering. That is, all of the ST354 Western Australia poultry isolates clustered and were distinct from the other ST354 strains. While many of the ST354 strains from poultry meat, humans, and dogs were intermingled, there was a cluster of poultry meat isolates from eastern Australia that all encoded 3 copies of *iha*. The extent of among-strain similarity according to variable

gene content was determined for the CC354 strains using a Principal Coordinates Analysis (PCO) (Jaccard similarity metric). The resulting similarity patterns mimicked those observed when using the core genome data (data not shown).

All CC354 strains (i.e., ST354 and ST3711) contained *sitA* and *usp*. The ST354 strains additionally contained *ibeA*, *iha*, *iucC*, and *iutA*, while the ST3711 strains did not. The Western Australia poultry meat isolates differed from other ST354 strains by containing *tia* and *hra*, and lacking *cah*. Other differences between ST354 and ST3711 isolates or between the Western Australian and eastern Australian isolates related to proteins of unknown function or phage related functions. Within ST354, no gene content feature categorically differentiated the eastern Australian poultry meat isolates from the human and companion animal isolates.

The plasmids hosted by CC354 isolates varied according to an isolate's ST membership and source. The ST3711 isolates and GNB 2829 (ST354) harboured an IncFIB plasmid encoding colicin M and containing a remnant of the colicin B activity gene. This plasmid also appeared to carry these strains' plasmid-borne resistance determinants. In contrast, the ST354 Western Australia poultry meat isolates hosted an IncQ conjugative plasmid that carried these strains' resistance determinants. Most of these isolates also harboured a colicin E1 plasmid. The balance of the ST354 strains appeared to harbour one of at least two different antimicrobial resistance plasmids: an IncQ plasmid or an IncFIA plasmid. Additionally, these eastern Australian isolates commonly also carried a colicin E1 plasmid in addition to the antimicrobial resistance plasmid.

The among-strain relationships based on the variable gene content of the inferred plasmid sequences (Fig. 3) broadly reflected the corresponding phylogenetic relationships, as inferred from core genome SNPs. The IncQ antibiotic resistance plasmids carried by the ST354 Western Australian poultry isolates were distinct from the IncQ plasmids hosted by the other ST354 isolates.

Discussion

In this study we defined the clonal structure of *E. coli* isolates of phylogroup F from diverse locales and ecologic sources in Australia, then compared clonal background with ecologic origin, locale, antimicrobial resistance, virulence gene content, and plasmid repertoire. We found that isolate source and isolation method had a large impact on the STs identified. For example, over half of the phylogroup F isolates recovered from water samples belonged to a single lineage (ST3637), representatives of which were recovered only from water samples. In contrast, the great majority of isolates belonging to ST59 or ST62 were from humans, were recovered without antimicrobial selection, and were FQ-susceptible. Reference to the Warwick MLST database supports the observation that isolates representing ST62 and ST59 are most likely to be recovered from humans, as all 26 examples of ST59 and all 42 examples of ST62 for which the database provides host data were from humans (strains from the present study excluded). Reference to the Warkwick MLST database shows that ST354 strains have been isolated humans and other animals, as was found in the present study

Although the biases in how the phylogroup F study isolates were sampled in regard to antimicrobial resistance phenotype preclude firm conclusions, it is also important to note that these isolates were not selected on the basis of their ST. Therefore, the results do suggest that phylogroup F isolates isolated from human faeces without antimicrobial selection are likely to be FQ-susceptible and to represent ST59 or ST62, while isolates belonging to CC354 are highly likely to be FQ-resistant, as all of the present CC354 isolates were FQ-resistant, including those recovered without antimicrobial selection.

The FQ-resistant ST3711 lineage of CC354 represented faecal isolates from native birds, chosen without respect to their FQ resistance, and were observed only in one wildlife rehabilitation centre in Western Australia, despite wild birds having been sampled from several other veterinary clinics and wildlife rehabilitation centres across Australia (Blyton et al. 2015). These were independent isolates, as all were collected from different individuals representing several avian species. The isolates are not identical, but are highly similar, which suggests a single 'clone' is circulating in this rehabilitation

centre.

Guo et al. (2015), who reported on a collection of FQ-resistant *E. coli* isolates recovered from the faeces or extra-intestinal sites of dogs, made a similar observation. Many of the phylogroup F dog isolates in their study came from the University Veterinary Teaching Hospital in Sydney Australia. All of the F isolates (n = 16) from that facility were from dog faeces and were members of ST354. Such an outcome suggests a ST354 'clone' was circulating in this care facility.

To date, few studies have determined the relative abundance of phylogroup F strains in faecal samples from Australia in the absence of antimicrobial selection. Those that have been reported indicate that phylogroup F strains typically represent about 7% of *E. coli* isolates from human faeces (Clermont et al., 2013; Blyton et al., 2014), but are less common in birds and mammals (1% in mammals [unpublished data] and 2% in birds [Blyton et al., 2015]). Therefore it would be unlikely that every dog arriving at a veterinary hospital or every bird arriving at a rehabilitation centre would all harbour strains belonging to the same ST (354 or 1377). In turn, this suggests strains belonging to CC354 have a propensity to persist and circulate in animal care facilities. Conditions favoring selection and maintenance of FQ-resistant strains do exist in Australian animal care facilities, as the fluoroquinolone enrofloxacin is commonly used to treat injured wildlife, and both enrofloxacin and marbofloxacin are registered for the treatment of companion animals (Gillett, 2010).

On average, phylogroup F strains are unlikely to possess virulence traits associated with extra-intestinal infection, however isolates belonging to ST59 and ST62 are an exception. Strains belonging to these STs are responsible for extra-intestinal infection (this study), harbour significantly more extra-intestinal virulence genes than most other phylogroup F isolates and, in particular, have a virulence gene profile linked to the ability to cause urinary tract infection. CC354 isolates likewise are also capable of causing extra-intestinal infection, but they are less likely than ST59 or ST62 isolates to harbour the genes typically associated with extra-intestinal infection, suggesting that they may

carry as-yet-unrecognised traits that enhance a strain's ability to cause extra-intestinal infection.

Some of the characteristics observed for the phylogroup F lineages ST59, ST62, and ST354 and ST3711 are mirrored by certain phylogroup B2 STs. Specifically, STs 95, 73, and 131 are frequently encountered, human-associated STs known to cause extra-intestinal infection (Riley, 2014). However, ST95 and ST73 strains are unlikely to be FQ-resistant, and generally encode few other resistant determinants, while ST131 strains are typically FQ-resistant and resistant to other antibiotics (Banerjee et al., 2013b; Tchesnokova et al., 2013). ST131 strains are also observed more frequently in environments such as hospitals and age-care facilities than are strains belonging to STs 95 and 73 (Banerjee et al., 2013a, 2013b). STs 59, 62, and 354 strains are frequently encountered phylogroup F strains also known to cause extra-intestinal infection. STs 59 and 62 are unlikely to be FQ resistant or resistant to other antimicrobials, while FQ resistance is common in ST354. The evidence also suggests that ST354 is more likely to be encountered in care facilities.

It is not apparent why isolates from STs 95, 73, 59, or 62 should be less likely to be FQ-resistant than those from STs 131 or 354. It seems unlikely that this dichotomy is due to differences in exposure to FQs, as rates of faecal carriage of all of these STs are broadly similar (within a phylogroup), and all these STs are capable of causing extra-intestinal infection. To argue otherwise would be to assume that hosts harbouring STs 95, 73, 59, or 62, intestinally or extra-intestinally, are less likely to be prescribed FQs compared to hosts that harbour STs 131 or 354. This may be true, if STs 131 and 354, for some non-antibiotic reason, associate preferentially with elderly, debilitated, and hospitalized or institutionalized hosts, who in turn are more likely than others to receive antibiotics. However, if all these STs are exposed to FQs at broadly the same frequency, then this in turn would suggest that the acquisition of FQ resistance is dependent on the genomic background of the bacterial host and that some genomic backgrounds are either pre-adapted to or incompatible with the evolution of FQ resistance. Further experiments are be required to determine if the ability of ST131 and CC354 isolates to

establish and maintain themselves in health care environments relates directly to the evolution of FQ resistance or is due to traits that may predispose these strains to evolving FQ resistance.

The use of antimicrobials in animals destined for human consumption is of considerable concern (Collignon, 2015). In Australia, fluoroquinolones cannot be administered to food-producing animals (Cheng et al., 2012) and Australia has extremely low prevalence of FQ-resistant Enterobacteriaceae among cattle, pigs, and sheep (Abraham et al., 2014a; 2014b, Abraham et al., 2015). However, FQ-resistant *E. coli* ST354 strains have been recovered from poultry meat in both Western Australia and the Australian Capital Territory (Ingram et al., 2013; this study). In eastern Australia, ST354 isolates from humans, companion animals and poultry meat are closely related in terms of both their core genomes and their variable gene content, and at the population level are indistinguishable. Indeed, poultry meat isolate W2-68 is virtually identical to several companion animal isolates. However, it cannot be concluded whether the presence of this strain in the poultry meat was due to its presence in the bird throughout the production cycle or contamination during processing and distribution of the poultry meat.

Although the other ST354 poultry meat isolates from eastern Australia were similar to isolates from humans and companion animals, they were not identical. This was most apparent for the cluster of poultry meat isolates that contained three copies of the adhesion-related gene *iha*, which were the only phylogroup F strains found to contain three copies of this gene. Further sampling will be required to determine if FQ-resistant ST354 strains containing three copies of *iha* can be recovered from humans or companion animals living in eastern Australia. Investigation and analysis of more ST354 isolates obtained directly from broilers is clearly required.

In conclusion, although group F is one of the least common *E. coli* phylogroups in Australia, it contains several lineages capable of causing extra-intestinal infection in humans, companion animals, and wild birds. Clonal complex 354 not only is a cause of

extra-intestinal infection, but is also highly likely to be FQ-resistant, and can be isolated from poultry meat products. Further studies are required to determine the prevalence and dissemination of FQ-resistant CC354 clones in Australia among community-dwelling humans, companion animals, and poultry, and the extent to which, within phylogroup F, FQ resistance is restricted to particular lineages.

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Table 1. Source and site of isolation of the phylogroup F isolates characterised in the present study.

Isolate Source	Site of isolation	Number of Isolates
Human	intestinal biopsy	4
	extra-intestinal	14
	faeces	7
Domestic dog	extra-intestinal	9
	faeces	1
Wild mammal	faeces	4
Chicken	faeces	2
	meat	17
Wild bird	faeces	16
Water		10
Unknown	unknown	3

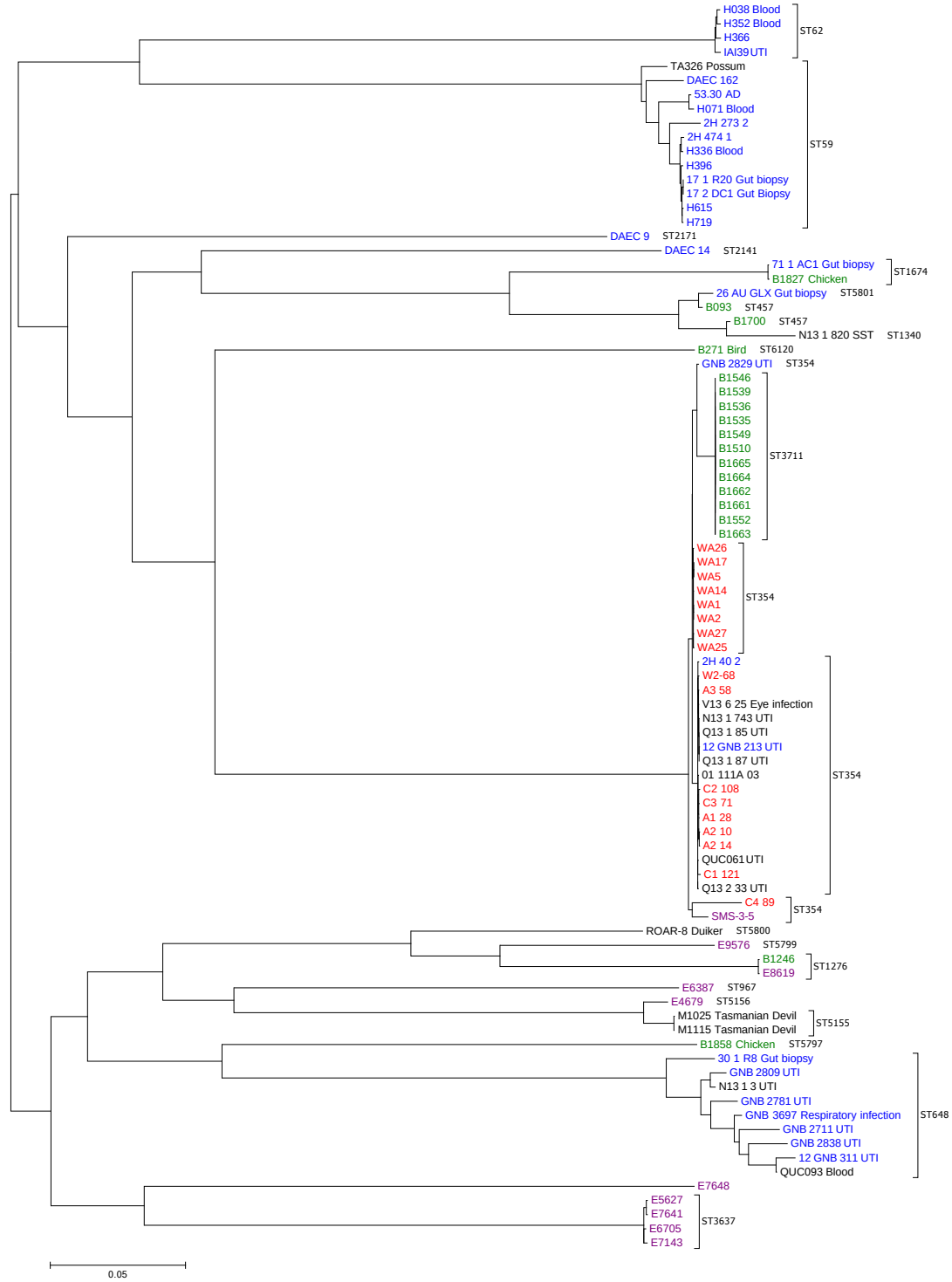

Fig 1. Phylogenetic relationships among 87 phylogroup F strains based on core genome SNPs as inferred using PhyML (Guindon et al., 2010), together with a general time reversible model of evolution. Bootstrap support values were 100% for all primary nodes. Green = native birds; red = Western Australia (strains with a WA prefix) or Australian Capital Territory poultry meat; black = non-human animals; blue = human; purple = water. Specimen type or source is listed for all non-fecal, non-water isolates.

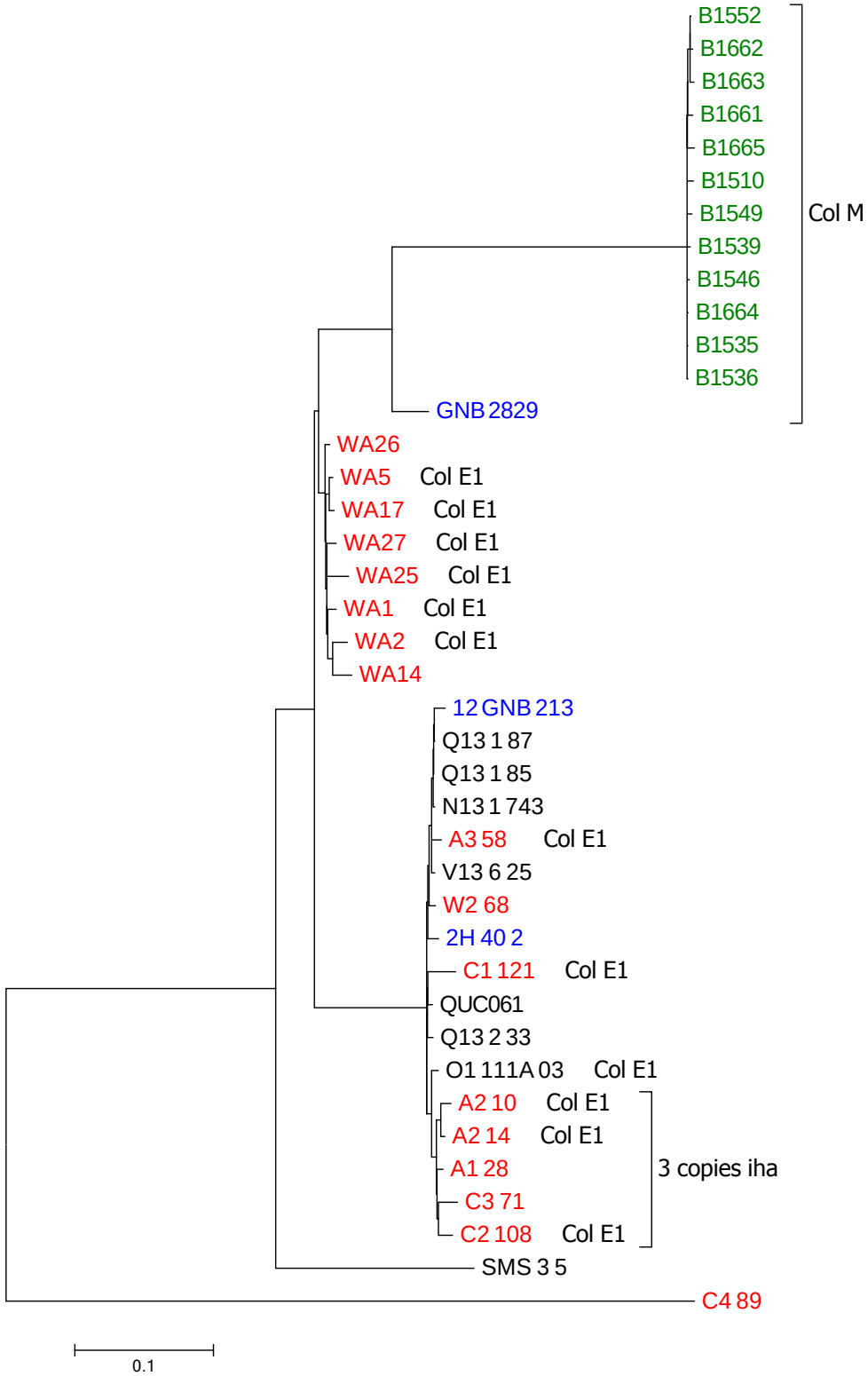

Fig 2. Phylogenetic relationships among CC354 strains based on core genome SNPs as inferred using PhyML (Guindon et al., 2010), together with a general time reversible model of evolution. Bootstrap support values were 100% for all primary nodes. The presence of known colicin plasmids in a strain is also indicated. Green = native birds; red = Western Australia (strains with a WA prefix) or Australian Capital Territory poultry meat; black = non-human animals; blue = human.

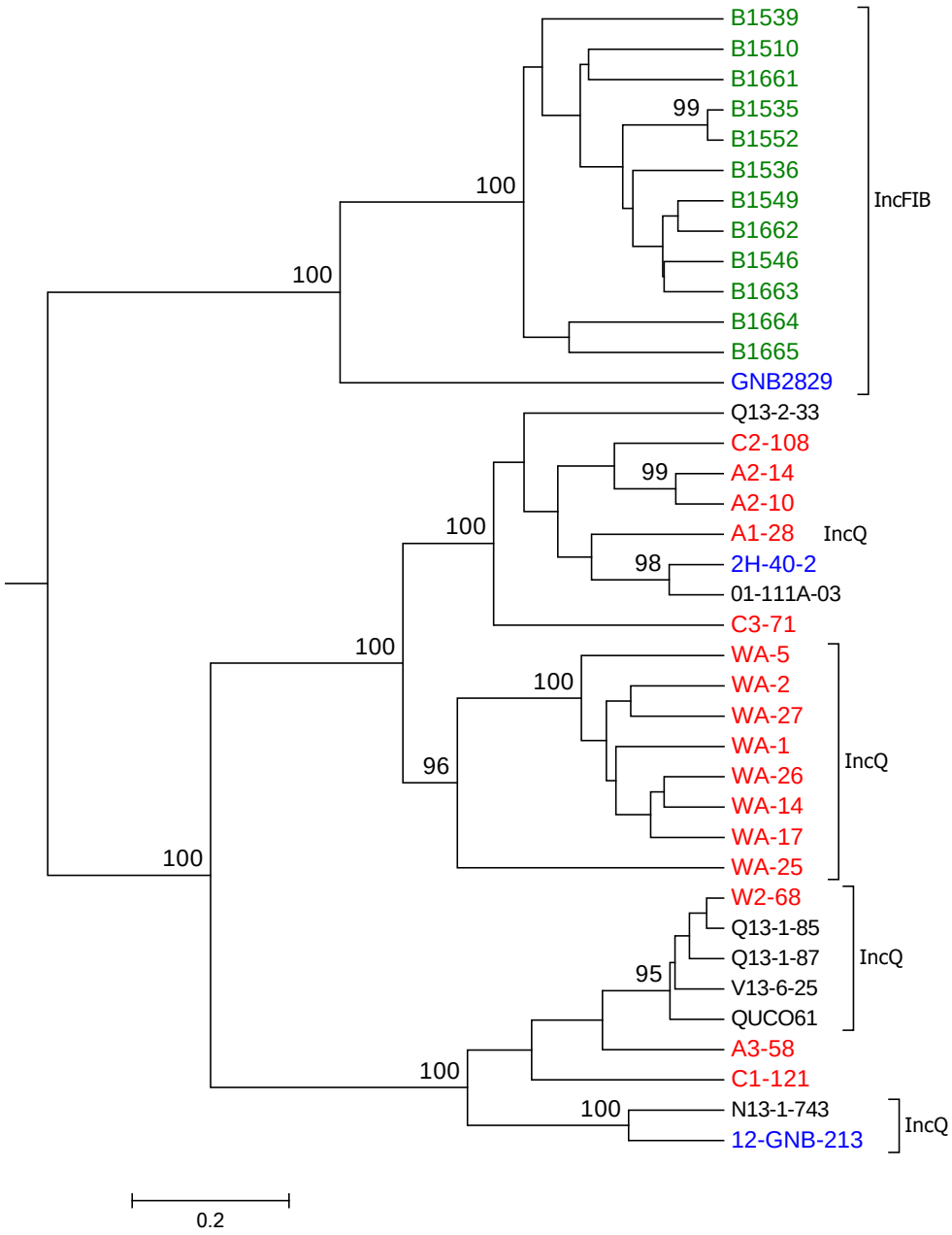

Fig 3. UPGMA tree based on the variable gene content of the antimicrobial resistance plasmids inferred to be present in the CC354 isolates. When no incompatibility group is defined, the incompatibility group of the plasmid carrying the antimicrobial resistance genes could not be unambiguously determined. Green = native birds; red = Western Australia (strains with a WA prefix) or Australian Capital Territory poultry meat; black = non-human animals; blue = human.